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(71) Applicant *(for all designated States except US)*: MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK [US/US]; One Gustave L. Levy Place, New York, NY 10029-6574 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BONA, Constantin [US/US]; 333 E. 55th Street, New York, NY 10022 (US). BRUMEANU, Teodor-Doru [CA/US]; Apartment 5K, 333 E. 93rd Street, New York, NY 10128 (US).

(74) Agents: CLARK, Richard, S. et al.; Brumbaugh, Graves, Donohue & Raymond, 30 Rockefeller Plaza, New York, NY 10112 (US).

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(57) Abstract

The present invention relates to pegylated proteins, including modified immunoglobulin molecules mildly derivatized with polyethylene glycol. Such "pegylated" immunoglobulins may be used to produce an enhanced immune response in the absence of adjuvant. In particular embodiments, the present invention relates to proteins conjugated to polyethylene glycol via carbohydrate residues.

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Description

PEGYLATED MODIFIED PROTEINS

1. INTRODUCTION

The present invention relates to pegylated proteins, including modified immunoglobulin molecules mildly derivatized with polyethylene glycol. Such "pegylated" immunoglobulins may be used to produce an enhanced immune response in the absence of adjuvant. In particular embodiments, the present invention relates to proteins conjugated to polyethylene glycol via carbohydrate residues.

2. BACKGROUND OF THE INVENTION

Polyethylene glycol ("PEG") is non-toxic, non-immunogenic, and is approved by the United States Food and Drug Administration for internal use in humans. It has been reported that covalent binding of monomethoxypolyethylene glycol ("mPEG") to proteins improves their solubility and half-life without significantly altering their biologic activity (Katre et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:1487; Suzuki et al., 1984, Biochim. Biophys. Acta 788:248; Kitamura et al., 1990, Biochem. Biophy. Res. Commun. 3:1387; Kitamura et al., 1991, Cancer Res. 51: 4310). For example, bovine serum catalase mildly derivatized with mPEG was found to retain full enzymatic activity in vitro (Abuchowski et al., 1977, J. Biol. Chem. 252:3582). Thereafter, various enzymes derivatized with mPEG were used for enzyme substitution and cancer therapy (Abuchowski et al., 1984, Cancer Biochem. Biophys. 7:175). Proteins of therapeutic use such as recombinant interleukin 2 ("rIL-2") were also derivatized with mPEG and showed increased potency to stimulate specific CTL activity in a murine sarcoma model (Katre et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:1487).

Mild conjugation to PEG ("pegylation") was used to increase the half-life of tumor-specific intact immunoglobulin molecules and $F(ab')_2$ fragments without significant alteration of the binding capacity to tumor antigens (Kitamura et al., 1990, Biochem. Biophy. Res. Commun. 3:1387). Pegylated anti-tumor antibodies were able to enhance tumor localization (Kitamura et al., 1991, Cancer Res. 51: 4310). However, tolerance to xenogeneic immunoglobulin determinants was achieved when the immunoglobulins were heavily derivatized with mPEG 5000 (Wilkinson et al., 1987, J. Immunol. 139:326). Furthermore, it was shown that synthetic self-peptides derivatized with mPEG were able to suppress the production of autoantibodies to acetylcholine receptor and to prevent experimental myasthenia gravis in mice (Atassi et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:5852).

The increase in half-life of pegylated immunoglobulins was correlated with resistance to proteolytic enzymes and with low clearance rate through the kidney. Indeed, when seven percent of the primary amines of human immunoglobulin were substituted with mPEG, the conjugates were resistant to trypsin digestion, and at 14 percent substitution they were resistant to pepsin digestion (Cunningham-Ruddles et al., J. Immunol. Methods 152:177). This indicated that pegylated immunoglobulin may be used in oral administration in the treatment of some gastrointestinal diseases.

3. SUMMARY OF THE INVENTION

The present invention relates to proteins which are covalently conjugated to polyethylene glycol ("pegylated"). In a first set of embodiments, the present invention provides for pegylated immunoglobulins. For example, immunoglobulin containing an immunogenic heterologous peptide corresponding to a T cell or a B

cell epitope may be pegylated to increase the half-life of the immunoglobulin without substantially decreasing the biological activity of the immunoglobulin molecule or the immunogenicity of the heterologous peptide. Such pegylated immunoglobulins containing heterologous peptide may be used to produce an enhanced immune response to the heterologous peptide in a subject. Such pegylated immunoglobulins may also be used to elicit an immune response to the heterologous peptide in a subject in the absence of adjuvant.

In further embodiments, the present invention provides for proteins that are conjugated to polyethylene glycol via carbohydrate residues, and for methods of producing such "glycopegylated" proteins. Proteins which may be pegylated in this manner include, but are not limited to, immunoglobulin, antigenic proteins, therapeutic proteins, and proteins used for diagnostic purposes.

4. DESCRIPTION OF THE FIGURES

Figure 1. *Blood clearance of native and pegylated Ig-HA.* Mice (three per group) were injected intravenously with ^{125}I-labeled Ig-HA or Ig-HA-mPEG. The mice were rested for 15 minutes to allow uniform distribution of radiolabeled material and then blood samples were collected at various intervals of time. Radioactivity content of total blood volume was estimated and considered as the total radioactivity injected (TRI). Blood samples were collected at indicated times and total residual radioactivity (TRR) was estimated. The percent of residual activity was estimated according to the following formula [1-(TRR/TRI)] x 100. Each point represents the mean ± SD of three mice.

Figure 2. *In vivo proteolysis of native and pegylated Ig-HA.* Serum samples from mice injected

intravenously with ^{125}I-labeled Ig-HA or Ig-HA-mPEG (20 x 10^6 cpm/mouse) were collected at indicated times after injection and 5μl of serum was run on an 8-17% gradient polyacrylamide gel. Separated material was electrotransferred onto PVDF membranes and exposed to Kodak X-OMAT films. The left panel (A) shows the pattern of in vivo degradation, over time, of samples collected from a mouse injected with Ig-HA. The right panel (B) shows the pattern of in vivo degradation, from a mouse injected with Ig-HA-mPEG. Samples of ^{125}I labeled Ig-HA and Ig-HA-mPEG (5 x 10^4 cpm/5μl) were analyzed in parallel in the corresponding panel and used as controls (C) of non- degraded material prior to inoculation into mice.

Figure 3. *Tissue distribution of native and pegylated Ig-HA.* Groups of seven mice each (15-17 g weight) were injected intravenously with ^{125}I-labeled Ig-HA or Ig-HA-mPEG. The mice were rested for 15 min to allow uniform distribution of the labeled material and blood samples were collected. Fragments of visceral organs (liver (A); spleen (B); Kidney (C); lung (D)) were then resected at indicated times after injection. The tissue distribution of native or pegylated Ig-HA was estimated as index of distribution (% ID). Each point represents % ID for a mouse corresponding to the indicated time after injection.

Figure 4. *In vitro activation of HA110-120 specific LD1-24 T cell hybridomas by native and pegylated Ig-HA.* Graded amounts of chromatographically purified Ig-HA, Ig-HA-mPEG and Ig-NP-mPEG were incubated with 2PK3 antigen presenting cells ("APCs"; 10^4) and LD1-24 T hybridoma cells (2 x 10^4). Activation of LD1-24 T cells was assessed 48 hours later by measuring IL-3 production in the cell supernatant using IL-3 dependent DA-1 cells (15 x 10^4) and MTT assay. Each

point represents the mean of quadruplets plus or minus SD.

Figure 5. *In vivo priming of HA110-120 specific T cells by native and pegylated Ig-HA.* Four groups of seven mice each were immunized subcutaneously at the base of the tail and in the foot pads with Ig-HA or IgHA-mPEG in either saline or CFA. Ten days later the draining lymph nodes were collected and cells were cultured for 5 days in standard conditions with graded amounts of various antigens. [^{3}H]-thymidine was added for 18 hours and incorporation was measured. Panels (a) and (b) represent the [^{3}H]-thymidine incorporation by lymph node cells from mice immunized with Ig-HA in saline and CFA, respectively. Panels (c) and (d) represent the [^{3}H]-thymidine incorporation by lymph node cells from mice immunized with Ig-HA-mPEG in saline and CFA, respectively. In all cases stimulation in vitro was carried out with graded amounts (0.5, 1, 5 and 10μg/ml) of Ig-HA, Ig-NP, HA110-120 synthetic peptide, NP147-161 synthetic peptide or UV-inactivated PR8 virus. [^{3}H]-thymidine incorporation upon in vitro stimulation with concanavalin A ("ConA") for three days is also indicated. Each point represents the mean of quadruplet wells plus or minus SD, after subtraction of cpm obtained in the absence of antigen.

Figure 6. *Antibody response of mice immunized with native and pegylated Ig-V_3C.* Groups of three mice were immunized with either Ig-V_3C (open circles) or Ig-V_3C-mPEG closed circles), bled at indicated intervals of time and the titers of antibodies to V_3C peptide (panels a, b, c and d) and to human isotypic determinants (panels e, f, g and h) were determined. In panel a and c the mice were immunized subcutaneously with 100μg of either Ig-V_3C or Ig-V_3C-mPEG emulsified in CFA and boosted two weeks later with 50μg of antigens in Incomplete Freund's adjuvant (IFA).

Arrows indicate the days of immunization. The other protocols of immunizations were performed with either three injections i.p. of 100μg of antigen in saline at seven day intervals (panels b and f), or two injections intraperitoneally of 100μg of antigens in saline at seven day intervals (panels c and g), or a single injection intraperitoneally of 100μg of antigens in saline (panels d and h). The antibody titers were determined by RIA. Each point represents the mean plus or minus S.D. of three mice.

Figure 7. *Separation of Ig-HA-mPEG conjugates from unpegylated Ig-HA by HPLC on Q300 anion exchange column*. Panel (a) shows the elution profile of the material collected from peak 1 in Figure 10 and loaded onto the Q300 column. Panel (b) shows a control elution profile of a preparation of unpegylated Ig-HA.

Figure 8. *Detection of residual mPEG in AIgs-mPEG preparations by electrophoresis on Titan agarose Gel*. Samples collected at various steps of purification were run on duplicate Titan gels and either stained with Coomassie/Ponceau (R250) or precipitated with TCA. Lane 1, IgHA-mPEG conjugate after purification on anion-exchange HPLC; lane 2, unpegylated IgHA; lane 3, mPEG 5,000 (4×10^{-4}M); lane 4, IgHA-mPEG conjugate after first step of purification by gel filtration on AcA44 (not done in Coomassie staining).

Figure 9. *Analysis of homogeneity of AIg-mPEG preparations by SDS-PAGE*. Samples collected subsequent to purification on anion-exchange HPLC were analyzed on 4-15% polyacrylamide gradient PhastGels (Pharmacia) under nonreducing conditions. Lanes 1 and 4, unpegylated Ig-HA and Ig-V_3C, respectively; lanes 2 and 4, Ig-HA-mPEG and Ig-V_3C-mPEG, respectively (content of peak 2 in Figure 8); and lanes 3 and 6 highly derivatized Ig-HA and Ig-V_3C (content of peak 1 in Figure 8).

Figure 10. Removal of hydrolyzed mPEG from AIg-mPEG preparation by size exclusion chromatography. AcA44 Ultrogel column (80 x 1.6 cm) was calibrated at 0.4 ml/min flow rate with molecular weight markers (Pharmacia, LKB) and then loaded with 10 mg of IgHA-mPEG preparation. The column was equilibrated with either 0.1M NH_4HCO_3 (panel a) or PBS (panel b). The peak labeled 1 contained the conjugates and peak 2 corresponds to free mPEG. A plus sign indicates the presence of free polymer as detected by Nessler's test.

Figure 11. Diagram of synthesis of Ig-Gal-PEG conjugates.

Figure 12. SDS-PAGE Analysis of Ig-Gal-PEG Conjugates. Chromatographic purified samples of the Ig-gal-PEG conjugates were analyzed on 4-20% polyacrylamide gradient gels under reducing and non-reducing conditions and stained with Coomassie R-250. Lanes 1 and 2 show reduced unpegylated and reduced pegylated human IgA from colostrum.

Figure 13. Isoelectric Focusing of Ig-Gal-PEG Conjugates. Chromatographic purified samples of the Ig-Gal-PEG conjugates were analyzed on PAG plates with a pH gradient between 3 and 10, as described in Section 8. Lane 1 shows the pI markers, pH 3-10, and lanes 2 and 3, pegylated and unpegylated human IgA from colostrum, respectively.

Figure 14. Titan Gel Electrophoresis of Ig-Gal-PEG Conjugates. Ig-Gal-PEG conjugates were analyzed by agarose electrophoresis as described in Section 8. Lane 1 represents glycopegylated human IgA and lane 2 represents native human IgA.

Figure 15. Estimation of "Gal Equivalents" on Native Igs. The Gal residues of human IgA from colostrum and rat IgG2b anti-mouse k chain mAb were determined by measuring GAO enzymatic activity as compared to known amounts of D-galactose, as described in

Section 8. Dotted plot represents GAO activity on D-galactose as a substrate. The corresponding Gal residues per molecule of Ig (nMole/Ig molecule) were calculated according to data obtained from GAO activity on the monosaccharide substrate (lower side of the abscissa). These data were then translated in number of "galactose equivalents" (upper side of the abscissa), knowing that 2.1 to 2.3 nMole of Gal residues corresponds to one Gal residue per molecule of Ig. As the rectangles indicate, the potential galactose sites of Igs for coupling to Gly-PEG were approximately four for the rat IgG2b anti-mouse k chain mAb and eight in the case of human IgA from colostrum.

Figure 16. Estimation of GAO Activity in the Presence of PEG. GAO activity was tested in the presence of different amounts of Gly-PEG 3,500 as described in Section 8. Slightly increased GAO activity was observed when PEG was co-incubated in the reaction mixture for 18 h at 37°C.

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) immunoglobulin molecules;

(ii) non-immunoglobulin molecules;

(iii) pegylation methods;

and

(iv) utilities of the invention.

5.1. IMMUNOGLOBULIN MOLECULES

In a first set of embodiments, the present invention relates to immunoglobulin molecules modified to contain a heterologous peptide, which are covalently conjugated to polyethylene glycol ("pegylated"). The heterologous peptide may be an immunogenic peptide or other biolocially active peptide.

The term "immunogenic", as used herein, is construed to mean that the heterologous peptide occurring in the modified immunoglobulin molecule is capable of eliciting a specific immune response. Preferably, the immunogenic heterologous peptide is a B cell epitope or a T cell epitope.

The invention is based, at least in part, on the discovery that the stability of such immunoglobulins and/or their ability to enhance the immune response is augmented when the modified immunoglobulins are mildly derivatized with polyethylene glycol ("pegylated").

For example, mild derivatization of the immunoglobulins containing a B cell or a T cell epitope may significantly increase the half-life of the immunoglobulin molecules without substantially decreasing the biological activity of the immunoglobulin molecule or the immunogenicity of the heterologous peptide. As used herein, "a substantial decrease" of the biological activity of an immunoglobulin molecule refers to the situation where the biological activity of the immunoglobulin has decreased to an extent where the immunoglobulin loses its ability to effectively deliver the B cell or a T cell epitope to immune cells. A "substantial decrease" in the immunogenicity of a B cell or a T cell epitope refers to the effective inability of the epitope to elicit a specific immune response.

Mild pegylation of immunoglobulin molecules containing a B cell epitope or a T cell epitope enhances the immunogenicity of the epitope and may obviate the requirement for adjuvants. For example, and not by way of limitation, the beneficial effects of pegylation of various immunoglobulins is set forth in Example Sections 6 and 7, below.

The present invention further provides for a method of pegylation which conjugates PEG to carbo-

hydrate residues of an immunoglobulin molecule, thereby minimizing structural distortion of the peptide backbone of the immunoglobulin or any heterologous epitopes comprised therein. A working example of such "glycopegylation" is set forth in Example Section 8, below.

The present invention may be applied to any immunoglobulin molecule, including human and nonhuman immunoglobulin molecules, immunoglobulin molecules containing both human and nonhuman portions, and immunoglobulins of any class (e.g. IgG, IgM, IgD, IgE, or IgA) and any specificity.

Such immunoglobulin molecules are modified to contain a heterologous peptide. For example, such a heterologous peptide may be a B-cell or T-cell epitope of an antigen, a cytokine, a toxin, or an idiotypic determinant, which has been covalently linked to the immunoglobulin molecule.

Such covalent linkage may be accomplished by genetic engineering techniques in which a nucleic acid sequence encoding the heterologous peptide is inserted into a nucleic acid sequence encoding the immunoglobulin molecule. Most preferably, the nucleic acid sequence encoding the heterologous peptide replaces a similarly sized sequence of a complementarity determining region ("CDR") of the immunoglobulin molecule, as set forth in United States patent application Serial No. 08/363,276. The engineered nucleic acid may then be incorporated into a suitable expression vector and expressed as a protein comprising an immunoglobulin molecule covalently linked to the heterologous peptide. More than one heterologous peptide (of the same or different variety) may be incorporated into the immunoglobulin by this method.

In one example, the genetically engineered immunoglobulin molecule contains a heterologous peptide, such as a T cell or a B cell epitope, in place of

a CDR loop of the immunoglobulin molecule, the CDR loop having been deleted and replaced with the heterogenous peptide. The CDR loop that is deleted and replaced with the heterologous peptide may be located in either the heavy or the light chain and may be CDR1, CDR2, or CDR3 loop.

The genetically engineered immunoglobulin molecule containing a heterologous peptide may be, for example, a humanized immunoglobulin molecule, an example of which is an immunoglobulin molecule genetically engineered to comprise the constant regions of a human immunoglobulin and the variable regions of a murine immunoglobulin.

Alternatively, the heterologous peptide may be linked to the immunoglobulin molecule by enzymatic means. For example, and not by way of limitation, the heterologous peptide may be linked to the immunoglobulin molecule via a carbohydrate residue of the immunoglobulin molecule, as set forth in United States Patent Application Serial No. 08/441,328, using a method analogous to the linkage of PEG to enzymatically oxidized carbohydrate residues of an immunoglobulin molecule, as exemplified in Section 8, below. In a preferred specific embodiment, heterologous peptide may be conjugated to enzymatically oxidized carbohydrate groups (e.g. galactose residues oxidized by galactose oxidase enzyme) by Schiff base formation, followed by reduction (e.g. with pyridine borane). Multiple heterologous peptides (of the same or different variety) may be linked to the immunoglobulin molecule by this method.

Virtually any peptide may be used according to the invention. Such peptides may comprise at least 2 and preferably at least five amino acid residues and may comprise immunogenic epitopes of antigens; suitable antigens include, but are not limited to, antigens

associated with pathogens, tumor cells, or "non-self" antigens with respect to a particular individual. Peptides may be biologically active themselves (for example, growth factors, toxins, immune mediators, differentiation factors etc.) or be portions of biologically active proteins. In specific, nonlimiting examples, the peptide may comprise all, or a portion, of IL-1 or tetanus toxoid.

In non-limiting embodiments, peptides which may be used according to the invention include B cell epitopes. The term "B cell epitope", as used herein, refers to a peptide, including a peptide comprised in a larger protein, which is able to bind to an immunoglobulin receptor of a B cell and participate in the induction of antibody production by the B cell.

For example, and not by way of limitation, the hypervariable region 3 loop ("V3 loop") of the envelope protein of human immunodeficiency virus ("HIV") type 1 is known to be a B cell epitope. Although the sequence of this epitope varies, the following consensus sequence, corresponding to residues 301-319 of HIV-1 gp120 protein, has been obtained: Arg-Lys-Ser-Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Phe-Tyr-Thr-Thr-Gly-Glu-Ile-Ile (SEQ ID NO:1).

Other examples of known B cell epitopes which may be used according to the invention include, but are not limited to, epitopes associated with influenza virus strains, such as Trp-Leu-Thr-Lys-Lys-Gly-Asp-Ser-Tyr-Pro (SEQ ID NO:2), which has been shown to be an immunodominant B cell epitope in site B of influenza HA1 hemagglutinin, the epitope Trp-Leu-Thr-Lys-Ser-Gly-Ser-Thr-Tyr-Pro (H3; SEQ ID NO:3), and the epitope Trp-Leu-Thr-Lys-Glu-Gly-Ser-Asp-Tyr-Pro (H2; SEQ ID NO:4; Li et al., 1992, J. Virol. 66:399-404); an epitope of F protein of measles virus (residues 404-414; Ile-Asn-Gln-Asp-Pro-Asp-Lys-Ile-Leu-Thr-Tyr; SEQ

ID NO:5) Parlidos et al., 1992, Eur. J. Immunol. 22:2675-2680); an epitope of hepatitis virus pre-S1 region, from residues 132-145 (Leclerc, 1991, J. Immunol. 147:3545-3552); and an epitope of foot and mouth disease VP1 protein, residues 141-160, Met-Asn-Ser-Ala-Pro-Asn-Leu-Arg-Gly-Asp-Leu-Gln-Lys-Val-Ala-Arg-Thr-Leu-Pro (SEQ ID NO:6; Clarke et al., 1987, Nature 330:381-384).

Still further B cell epitopes which may be used are known or may be identified by methods known in the art, as set forth in Caton et al., 1982, Cell 31:417-427.

In additional embodiments of the invention, peptides which may be used may be T cell epitopes. The term "T cell epitope", as used herein, refers to a peptide, including a peptide comprised in a larger protein, which may be associated with MHC self antigens and recognized by a T cell, thereby functionally activating the T cell.

For example, a peptide may comprise a T_h epitope, which, in the context of MHC class II self antigens, may be recognized by a helper T cell and thereby promote the facilitation of B cell antibody production via the T_h cell.

For example, and not by way of limitation, influenza A hemagglutinin (HA) protein bears, at amino acid residues 110-120, a T_h epitope having the amino acid sequence Ser-Phe-Glu-Arg-Phe-Glu-Ile-Phe-Pro-Lys-Glu (SEQ ID NO:7).

Other examples of known T cell epitopes include, but are not limited to, two promiscuous epitopes of tetanus toxoid, Asn-Ser-Val-Asp-Asp-Ala-Leu-Ile-Asn-Ser-Thr-Lys-Ile-Tyr-Ser-Tyr-Phe-Pro-Ser-Val (SEQ ID NO:8) and Pro-Glu-Ile-Asn-Gly-Lys-Ala-Ile-His-Leu-Val-Asn-Asn-Glu-Ser-Ser-Glu (SEQ ID NO:9; Ho et al., 1990, Eur. J. Immunol. 20:477-483); an epitope of

cytochrome c, from residues 88-103, Ala-Asn-Glu-Arg-Ala-Asp-Leu-Ile-Ala-Tyr-Leu-Gln-Ala-Thr-Lys (SEQ ID NO:10); an epitope of *Mycobacteria* heatshock protein, residues 350-369, Asp-Gln-Val-His-Phe-Gln-Pro-Leu-Pro-Pro-Ala-Val-Val-Lys-Leu-Ser-Asp-Ala-Leu-Ile (SEQ ID NO:11; Vordermir et al., Eur. J. Immunol. 24:2061-2067); an epitope of hen egg white lysozyme, residues 48-61, Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg (SEQ ID NO:12; Neilson et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:7380-7383); an epitope of *Streptococcus A* M protein, residues 308-319, Gln-Val-Glu-Lys-Ala-Leu-Glu-Glu-Ala-Asn-Ser-Lys (SEQ ID NO:13; Rossiter et al., 1994, Eur. J. Immunol. 24:1244-1247); and an epitope of *Staphylococcus* nuclease protein, residues 81-100, Arg-Thr-Asp-Lys-Tyr-Gly-Arg-Gly-Leu-Ala-Tyr-Ile-Tyr-Ala-Asp-Gly-Lys-Met-Val-Asn (SEQ ID NO:14; de Magistris, 1992, Cell 68:1-20). Still further T_h epitopes which may be used are known or may be identified by methods known in the art.

If the peptide comprises a T cell epitope, it may be desirable to provide a means for releasing the T cell epitope from the immunoglobulin so as to facilitate appropriate processing by an antigen presenting cell ("APC"). For example, it may be desirable to incorporate the T cell epitope into a larger peptide which comprises a site susceptible to cleavage by an enzyme typically present in lysosomes of APCs. As a specific example, the lipophilic quadruplet Ala-Ala-Ala-Leu (SEQ ID NO:15), that contains the cleavage site for cathepsins (Yonezawa et al., 1987, Arch. Biochem. Biophys 256:499), may be added to the T cell epitope. It is known that lysosomal cathepsins play an important role in processing of exogenous molecules.

Peptides for use according to the invention may be purified from natural sources, or may be recombinantly and/or chemically synthesized. They may con-

tain amino acid analogs, and may be detectably labelled.

Immunoglobulins may be covalently linked to polyethylene glycol ("pegylated") by any method known in the art for conjugating polyethylene glycol to a protein or to a carbohydrate (see Section 5.3, infra.

In preferred nonlimiting embodiments of the invention, following conjugation, the molar ratio of PEG residues per immunoglobulin is at least 50 to 1.

The chemically activated PEG (i.e., PEG rendered capable of covalent linkage by chemical means) used to pegylate the immunoglobulin molecules of the present invention may be any of the many activated forms of PEG known to those skilled in the art, including but not limited to activated mPEG 5000 or activated mPEG 3500. The particular activated PEG used may be of any of the molecular weights known to those skilled in the art. It is preferred that PEG activation be achieved via succinimide or triazine.

In a preferred, specific, nonlimiting embodiment of the invention, chemically activated PEG (i.e., PEG rendered capable of covalent linkage by chemical means) may be covalently linked to an immunoglobulin via lysine residues contained in the immunoglobulin, by the following method:

A 50-fold molar excess of 2,4,6-trichloro-s-triazine activated mPEG 5000, also known as 2-O-monomethoxypolyphenylene glycol 4,6-dichloro-S-triazine, or another size or activated form of PEG, may be added to a solution of the immunoglobulin to be conjugated in 0.1M tetraborate buffer, pH 9.6, stirred vigorously for four hours at room temperature (in one specific nonlimiting embodiment, at least 18°C), and then concentrated using an ultrafiltration/concentration apparatus having a 100,000 molecular weight cut off ("MWCO"). In a specific, nonlimiting embodiment, a

50-fold molar excess of 2,4,6-trichloro-s-triazine activated mPEG 5000 may be added to a solution of 10 mg of the immunoglobulin to be conjugated in 10 ml of 0.1M tetraborate buffer, pH 9.6, stirred vigorously for four hours at room temperature, and then concentrated to 1.5 ml in a CENTREX UF-2 ultraconcentrator of 100,000 MWCO (Schleicher & Schuell).

In pegylating an immunoglobulin molecule containing an immunogenic heterologous polypeptide corresponding to a T cell or a B cell epitope, it is preferred that the amount of pegylation be effective for increasing the half-life of the immunoglobulin molecule without substantially decreasing the biological activity of the immunoglobulin molecule or the immunogenicity of the heterologous peptide. Preferably, the amount of pegylation should be effective for increasing the immunogenicity of the heterogenous peptide without substantially decreasing the biological activity of the immunoglobulin molecule. It is also preferred that the amount of pegylation be effective for conferring to the immunoglobulin molecule an ability to elicit a immune response to the heterologous peptide in a subject, when the immunoglobulin molecule is introduced into the subject without an adjuvant.

In one example, when the pegylation occurs via epsilon amino groups of the lysine residues that are accessible for pegylation, approximately 4 to 10 % of all the accessible epsilon amino groups of lysine residues are pegylated. In the most preferred example, approximately 6 to 8 % of the accessible epsilon amine groups of lysine residues are pegylated.

The degree of mPEG derivatization may be estimated by fluorescamine assay as described in Cunningham-Ruddles et al., 1992, J. Immunol. Methods 152:177. In a specific, nonlimiting embodiment, graded amounts (e.g., 0.25, 0.5, and 1 microgram) of immuno-

globulin-mPEG conjugate may be dissolved in 25 microliters of PBS and mixed with an equal volume of fresh fluorescamine in acetone (150g/ml). Samples may then be incubated for 5 minutes at room temperature and then adjusted to 1 ml with PBS. The absorbance may then be measured at 475 nm emission versus 390 nm excitation and the percent of pegylated residues may be estimated by the formula:

$$1 - \{(OD_{475} \text{ Ig-mPEG} / OD_{475} \text{ native Ig}) \times 100\}$$

Preparations of PEG-immunoglobulin conjugates may then be purified to substantially remove unreacted PEG as well as any undesirable products of the conjugation process, such as residual adducts.

For example, and not by way of limitation, where PEG is conjugated to immunoglobulin via lysine residues using 2,4,6-trichloro-s-triazine activated mPEG 5000, as set forth above, the following two-step method of purification may be used. First, residual unreacted mPEG may be removed by, for example, size exclusion chromatography, ultrafiltration, or dialysis followed by either anion exchange chromatography, hydrophobic chromatography, or reverse phase chromatography., for example, but not by way of limitation, on an AcA44 Ultogel column which has been equilibrated with 0.1 M ammonium hydrogencarbonate, pH 8.5 and flow-rate of 0.4 ml/min. It is desirable to remove unreacted mPEG prior to any attempt to remove residual adducts by chromatography, as the presence of unreacted PEG could interfere with the removal of such adducts. As a specific, nonlimiting example, unreacted mPEG may be removed as follows. The products of conjugation reaction may be applied to an AcA44 Ultrogel filtration column (80 x 1.6 cm) which has been equilibrated with 0.1M NH_4HCO_3, pH 8.5 and a flow rate of 0.4 ml per minute. Fractions of eluate may then be collected at 4 minute intervals, dried by speed vacuum centrifugation

and resuspended in 0.5 ml of 5 mM sodium acetate, pH 5.0. Each fraction may then be analyzed for protein content by, for example, Biuret micro assay, and for the presence of free hydrolyzed mPEG by Nessler's reagent, as described in Wilkinson et al., 1987, Immunol. Lett. 15:17-22. Fractions of the conjugates which are found to be free of hydrolyzed mPEG may then be subjected to a second chromatography step to remove residual adducts. In this second step, fractions of conjugates found to be free of hydrolyzed mPEG may then be applied to a Q300 anion exchange HPLC column (or a similar anion exchange column) which has been equilibrated with 5 mM sodium acetate, pH 5 using a 45 minute linear gradient from 5 to 500 mM sodium acetate, pH 5, and a flow rate of 0.5 ml/min. Fractions from the Q300 column may then be dialyzed in Spectrapor bags with 75,000 MWCO against PBS and concentrated in CENTREX UF-2 tubes. Other methods, such as those set forth for removing unreacted PEG, could also be used to remove residual adducts. Fractions containing useful PEG-IG conjugates may be identified, for example, by fluorescamine assay, mass spectroscopy, proton NMR, or SDS-PAGE.

It may further be desirable to confirm the homogeneity of the purified immunoglobulin-PEG conjugates by SDS-PAGE under non-reducing conditions (Laemmli, 1970, Nature 227:680-685).

5.2. NON-IMMUNOGLOBULIN MOLECULES

The present invention further provides for pegylation of non-immunoglobulin molecules, where polyethylene glycol is conjugated to a non-immunoglobulin molecule via carbohydrate residues, using enzymatic methods as set forth in section 5.3., infra.

Examples of non-immunoglobulin molecules which may be pegylated in this manner include, but are not limited to, proteins which are naturally or arti-

ficially glycosylated. Proteins which are, or may be rendered, glycosylated include bacterial, viral, protozoal, yeast, funal or insect proteins, proteins expressed in cells of lower or higher vertebrates, receptor proteins, signal transductions proteins, cytokines, enzymes, membrane proteins, etc.

5.3. ENZYMATIC METHODS OF PEGYLATION

The present invention also provides for an enzymatic method which may be used to couple PEG to an immunoglobulin or non-immunoglobulin protein. According to this method, PEG may be linked to an oxidized carbohydrate residue (such method referred to as "glycopegylation") on a protein molecule thereby minimizing the distortion of peptide portions of the protein that may be the result of pegylating amino acid primary amines. Such an oxidized carbohydrate residue may be formed by reacting a glycosylated (e.g. natively glycosylated) immunoglobulin molecule with a sugar oxidase enzyme. In a particular, nonlimiting embodiment of the invention, immunoglobulin-Gal-PEG conjugates (Ig-Gal-PEG) may be synthesized by oxidizing a galactose (Gal) residue of an asialo- or desialylated Ig with galactose oxidase (GAO), and then forming a Schiff base between the C6-aldehyde group of the Gal residue and an amino terminal group of Gly-PEG, with concurrent reductive alkylation of the imidic bond by reduction with pyridine borane (PB). This method is diagrammed in Figure 11. Section 8, infra, provides a specific example of this method, in particular, the synthesis of human and mouse Ig conjugates with an amino acid derivative of PEG 3,400 (Gly-PEG).

5.4. UTILITIES OF THE INVENTION

In a first set of embodiments, the present invention provides for a method of enhancing an immune response toward a heterologous peptide in a subject, comprising administering, to the subject, an effective

amount of purified immunoglobulin molecule containing the heterologous peptide, wherein at least 5 percent of the lysine residues of the immunoglobulin molecule are covalently linked to polyethylene glycol.

The pegylated immunoglobulins of the present invention may be useful in the diagnosis and treatment of a wide variety of malignancies and viral infections.

Accordingly, the present invention provides for a method of treating a viral (or bacterial, protozoan, mycoplasmal, or fungal) infection comprising administering, to a subject in need of such treatment, an effective amount of a composition comprising pegylated immunoglobulin, as described in the preceding sections, containing a heterologous peptide which comprises an epitope of a viral, bacterial, protozoan, mycoplasmal, or fungal antigen. The term "treating" as used herein refers to an amelioration in the clinical condition of the subject, and does not necessarily indicate that a complete cure has been achieved. An amelioration in clinical condition refers to a prolonged survival, a decreased duration of illness, or a subjective improvement in the quality of life of the subject.

The present invention provides for a method of enhancing an immune response directed toward a viral, protozoan, mycoplasmal, bacterial or fungal pathogen, in a subject in need of such treatment, comprising administering, to a subject in need of such treatment, an effective amount of a composition comprising pegylated immunoglobulin, as described in the preceding sections, containing a heterologous peptide which comprises an epitope of a viral, protozoan, mycoplasmal, bacterial, or fungal antigen. The phrase "enhancing an immune response" refers to an increase in cellular and/or humoral immunity. In preferred embodiments, the amount of cellular and/or humoral immunity

is increased in the subject by at least 25 percent. Such an enhanced immune response may be desirable during the course of infection, or before infection may have occurred (for example, in the context of a vaccine).

The present invention also provides for a method of treating a malignancy or other neoplasm comprising administering, to a subject in need of such treatment, an effective amount of a composition comprising pegylated immunoglobulin, as described in the preceding sections, containing a heterologous peptide which comprises an epitope of an antigen associated with a malignancy or neoplasm. In addition to the definition of "treating" set forth above, tumor regression, such as a decrease in tumor mass or in the number of metastases, of preferably at least 25 percent would be considered "treating".

Further, the present invention provides for a method of enhancing an immune response directed toward a malignancy or other neoplasm, in a subject in need of such treatment, comprising administering, to a subject in need of such treatment, an effective amount of a composition comprising pegylated immunoglobulin, as described in the preceding sections, containing a heterologous peptide which comprises an epitope of an antigen associated with a malignancy or neoplasm.

As with all pharmaceutical compositions, the effective amounts of the pegylated immunoglobulins of the invention may be determined empirically. Factors to be considered include the condition to be treated, whether or not the antibody will be complexed with or covalently attached to a toxin, route of administration for the composition, i.e. intravenous, intramuscular, subcutaneous, etc., and the number of doses to be administered. Such factors are known in the art and it

is well within the skill of physicians to make such determinations without undue experimentation.

The pegylated immunoglobulins of the invention offer a number of in vitro utilities, and the invention provides for methods based on such utilities. Such utilities include, for example, radioimmunoassays and ELISA assays, and fluorescence activated cell sorting.

In a second set of embodiments, the half-life of non-immunoglobulin molecules may be extended by enzymatic pegylation of carbohydrate residues on the non-immunoglobulin molecules. For example, but not by way of limitation, the half-life of a viral protein may be extended by such pegylation, such that a vaccine comprising the viral protein may become more antigenic. As a second example, an enzyme may be rendered more stable by such pegylation, so that the enzyme may be used more effectively in either enzyme supplementation therapy in vivo, or in diagnostic or therapeutic applications.

The following examples are meant to illustrate but not limit this invention.

6. EXAMPLE: DERIVATIZATION OF CHIMERIC IMMUNOGLOBULINS WITH PEG

6.1. MATERIALS AND METHODS

Mice. Six week old BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Antigens. The synthetic peptide HA110-120 (SFERFEIFPKE; SEQ ID NO:7) corresponds to amino acid residues 110-120 of the hemagglutinin (HA) of PR8 influenza A virus (Zaghouani et al., 1993, Science 259:224), NP 147-161 (TYQRTRALVRTGMDP; SEQ ID NO:16) corresponds to residues 147-161 of the nucleoprotein (NP) of PR8 influenza A virus (Zaghouani et al., 1992,

J.Immunol., 148: 3604) and V_3C peptide (RKSIHIGPGRAFYTTGEII; SEQ ID NO:1) corresponds to a consensus sequence predicted from the comparison of the V_3 cysteine bridged loop sequences of gp120 envelope proteins of 245 HIV-1 isolates. Peptides were synthesized and RP-HPLC purified at IAF Biochem (Laval, Quebec, Canada). KLH and BSA conjugates of the synthetic peptides were prepared using standard techniques.

Chimeric Immunoglobulins. Ig-HA and Ig-V_3C are genetically engineered chimeric immunoglobulins. Ig-HA is a BALB/c IgG2b expressing, in the CDR3 loop, a T cell epitope (consisting of the HA110-120 peptide) from the HA of PR8 influenza A virus. Ig-NP is also a BALB/c IgG2b expressing, in the CDR3 loop, the NP147-161 CTL-epitope consisting of the NP147-161 peptide. Ig-V_3C is a chimeric immunoglobulin comprising mouse variable and human γ1/k constant regions, and containing, within the heavy chain CDR3 loop, a B cell epitope from the V_3 cysteine bridged loop of the envelope protein of HIV-1. Ig-W is a chimeric immunoglobulin comprising wild type mouse V_H and V_L variable regions and human γ1/k constant regions, which was used as a control for Ig-V_3C. Ig-HA and Ig-NP were affinity purified from cell culture supernatant on a rat anti-mouse k chain-sepharose column. Ig-V_3C and Ig-W were affinity purified on a mouse anti-human k chain antibody (HP605)-sepharose column.

Virus. PR8 influenza A virus was prepared from allantoic fluid of embryonated eggs on a sucrose gradient. An UV inactivated PR8 virus was used in these experiments.

Derivatization of chimeric Ig with mPEG. Ig-HA, Ig-NP, and Ig-V_3C (see above) were derivatized with 2,4,6-trichloro-s-triazine activated mPEG 5,000 (Sigma) and purified to homogeneity. Briefly, a 50 times molar

excess of mPEG was added to 10 mg of chimeric immunoglobulin in 10 ml of 0.1M tetraborate buffer, pH 9.6. The mixture was stirred vigorously for four hours at room temperature and the preparations were concentrated to 1.5 ml in CENTREX UF-2 ultraconcentrators of 100,000 MWCO (Schleicher & Schuell). The residual unreacted mPEG was removed by size exclusion chromatography on an AcA44 Ultrogel column and homogeneous populations of Ig-mPEG with 6-8% derivatized lysines were isolated by anion exchange HPLC on Q300 column (for details of procedure, see Example Section 7, below). The degree of mPEG derivatization was estimated by fluorescamine assay as described in Cunningham-Rundles et al., 1992, J.Immunol. Methods 152:177. Briefly, graded amounts (0.25, 0.5 and 1μg) of Ig-HA-mPEG, Ig-V_3C-mPEG conjugates and native Ig-HA or Ig-V_3C, were dissolved in 25 μl of PBS and mixed with an equal volume of fresh fluorescamine in acetone (150 g/ml). Samples were incubated for 5 min at room temperature and then adjusted to 1 ml with PBS. The absorbance was then measured at 475 nm emission versus 390 nm excitation and the percent of pegylated lysines was estimated according to the following formula: 1-[(OD_{475} Ig-mPEG / OD_{475} native Ig)x100]. The homogeneity of the conjugates was confirmed by SDS-PAGE and agarose electrophoresis.

Blood circulation. Ig-HA and Ig-HA-mPEG preparations (100μg in 100μl of PBS) were radiolabeled with ^{125}I using the chloramine method and the free iodine was then removed by gel filtration on PD10 columns (Pharmacia). Groups of three BALB/c mice were injected intravenously with 200μl of PBS containing 20 x 10^6 cpm (4μg) of ^{125}I-labeled native Ig-HA or Ig-HA-mPEG. Fifteen minutes later (time required for uniform distribution of labeled Ig) the mice were bled and the radioactivity in 20μl of serum was counted. Total

radioactivity per mouse was estimated on the basis that 7.3% of body weight is blood and 55% of the blood volume is serum (Klibanov et al., 1991, Biochim. Biophys.Acta 1062: 142). The radioactivity estimated at 15 min was considered as the total radioactivity injected. To investigate the clearance of Ig-HA and Ig-HA-mPEG, mice were bled 3, 6, 24, 48, 72, and 168 hours after injection. Blood clearance rates were expressed for each group as percentage of residual radioactivity at various intervals of time.

Organ distribution. To study the tissue distribution of the native Ig-HA and Ig-HA-mPEG conjugates, two groups of seven mice each (15-17g weight) were injected intravenously with 200μl PBS containing 20 x 10^6 cpm of ^{125}I-labeled native Ig-HA or Ig-HA-mPEG. Mice were bled after 15 min and the blood radioactivity was estimated as above. Fragments of liver, spleen, lung and kidney were then resected by 3, 6, 24, 48, 72, and 168 hours after inoculation and the radioactivity was measured in a γ counter. Tissue distribution was expressed as index of distribution (%ID) of ^{125}I-Ig-HA or ^{125}I-Ig-HA-mPEG. ID represents the percentage of radioactivity per gram of wet organ versus the radioactivity measured in blood at 15 min after injection (Kitamura et al., 1990, Biochem. Biophys. Res. Commun.: 1387).

Western blot analysis. To investigate in vivo proteolysis of the native and pegylated Ig-HA, blood samples from mice injected with ^{125}I-Ig-HA or ^{125}I-Ig-HA-mPEG as for the clearance studies, were analyzed by SDS-PAGE under non- reducing conditions. Briefly, serum samples (5μl) collected at various intervals of time were electrophoresed on 8 to 17% polyacrylamide gradient gels (ExcelGels, Pharmacia) for 2 hours, at 150 volts and 40°C using a Multiphore II system (Pharmacia). Separated proteins were electro-

transferred from the gel onto PVDF membranes (0.22μ, Millipore, Waters Co.) for 30 minutes at 450 mAmps. Membranes were dried and exposed to Kodak X-OMAT films overnight at -70°C.

In vitro T cell proliferation assay. 2PK3 B lymphoma cells (H-2d) were used as antigen presenting cells ("APCs"). Irradiated (2,200 rads) APCs (10^4) were incubated for 48 hours in round bottom 96-well plates with graded amounts of Ig-HA, Ig-HAmPEG or Ig-NP-mPEG, and 2 x 10^4 of HA110-120 specific T hybridoma cells LD1-24 (Haberman et al., J. Immunol. 145:3087). Supernatants were then harvested and IL-3 production, as a measure of T cell proliferation, was determined using 1.5 X 10^4 of IL-3 dependent DA- I cells and the calorimetric MTT assay (Zaghouani et al., Science 259:224, Mosmann, 1983, J. Immunol. Methods 65: 55).

In vivo T cell proliferation assay. The proliferative response of lymph node cells from BABL/c mice immunized with Ig-HA or Ig-HA-mPEG were assessed for [^{3}H]-thymidine incorporation. Four groups of seven mice each were immunized subcutaneously at the base of the tail and in the foot pads with 100μg of Ig-HA or Ig-HA-mPEG emulsified in either 200μl of PBS or 200μl of PBS/CFA (1:1 v/v). Ten days later, the draining lymph nodes were collected and the cells were cultured in flat bottom 96-well microtiter plates (2 x 10^5 cells per well) with 0.5, 1, 5, and 10μg/ml of HA110-120, NP147-161, Ig-HA, Ig-NP, UV-inactivated PR8 virus, ConA (1μg/ml) or no antigen. Cultures were set up in quadruplets from pooled lymph node cells, incubated under standard conditions for 5 days (or 3 days in the case of ConA), and pulsed for the last 18 hours with 1 μCi of [^{3}H]-thymidine per well. The cells were then harvested and the radioactivity was measured in a β scintillation counter. The mean of cpm obtained in the

absence of antigen was subtracted from the mean of cpm obtained in the presence of antigens.

Radioimmunoassay. Detection of the anti-V_3C peptide antibodies in mice sera was carried out by radioimmunoassay as follows: microtiter plates were coated with 50µl of PBS containing 5µg/ml of V_3C peptide coupled to BSA (V_3C-BSA), BSA, or NP147-161-BSA for 18 hours at 40°C. The plates were extensively washed with PBS and then blocked with 3% BSA in PBS at room temperature. After 4 hours the plates were washed with PBS and serial serum dilutions (50µl) in 1% BSA-PBS were added. The plates were incubated for 2 hours at 37°C. After washing with PBS-0.05% Tween 20, bound antibodies were revealed by incubating the plates for 2 hours at room temperature with 5×10^4 cpm of ^{125}I-rat anti-mouse k chain mAb. Plates were then washed and bound radioactivity was measured in a γ counter. The amount of anti-V_3C antibodies in mice sera was estimated by extrapolation on a standard curve constructed with affinity purified mouse polyclonal anti-V_3C antibodies. These polyclonal anti-V_3C antibodies were obtained from BALB/c mice immunized with V_3C-KLH conjugate (100 µg) in CFA and then boosted weekly, three times with V_3C-KLH conjugate (50µg) in Incomplete Freund adjuvant (IFA). The antibodies were affinity purified on a V_3C-BSA-sepharose column.

Detection of anti-human isotypic antibodies in mice sera was carried out by radioimmunoassay as follows: microtiter plates were coated with 50µl of PBS containing 2µg/ml of Ig-W for 18 hours at 40°C. The plates were then treated as above. The amount of mouse anti-human Ig antibodies was estimated by extrapolation on a standard curve constructed with a stoichiometric mixture of mouse anti-human γ1 mAb (Zymed Laboratory) and HP6053 mouse anti-human kappa mAb (ATCC).

6.2. RESULTS

Preparation of homogeneous populations of pegylated chimeric immunoglobulins.

Ig-HA and Ig-V_3C were mildly pegylated and homogeneous conjugates with 6-8% mPEG substitution of the lysine residues were purified as described in Section 7, below. The conjugate preparations were rendered free of residual adducts such as free hydrolyzed mPEG and highly conjugated or unconjugated immunoglobulin by size exclusion chromatography performed on AcA44 Ultrogel column followed by anion-exchange HPLC on Q300 column. Homogeneous populations of Ig-HA-mPEG and Ig-V_3C-mPEG conjugates with 6-8% mPEG substituted lysine residues as determined by fluorescamine assay, were used for further investigations.

Blood clearance and tissue distribution of native and pegylated chimeric immunoglobulins.

Clearance rates. The clearance rates of Ig-HA-mPEG from blood circulation were analyzed in parallel to those of native Ig-HA. ^{125}I-labeled Ig-HA-mPEG and Ig-HA were injected intravenously into BALB/c mice and the radioactivity was measured in blood samples collected at various intervals of time. As depicted in Figure 1, native Ig-HA was cleared at 48 hours after injection while lg-HA-mPEG persisted in peripheral blood up to 7 days. Ig-HA-mPEG showed a two phase clearance characterized by an initial rapid decline followed by a much slower decline.

Resistance to <u>in vivo</u> proteolysis. Serum samples from mice injected with ^{125}I-labeled native Ig-HA or Ig-HA-mPEG were collected at various intervals of time and analyzed by Western blot analysis. Both native and pegylated Ig-HA showed significant degradation between 6 and 24 hours after injection. By 48

hours, the majority of the proteolytic products were cleared from the blood, and by 72 hours intact immunoglobulin molecules were detected only in samples from mice injected with Ig-HA-mPEG (Figure 2).

Tissue distribution. We carried out kinetic studies to estimate the amount of radioactivity in various tissues at various intervals of time after injection of the radiolabeled conjugates. The tissue distribution of ^{125}I-Ig-HA and ^{125}I-Ig-HA-mPEG was studied in liver, spleen, lung and kidney resected at 0.25, 3, 6, 24, 48, 72 and 168 hours after injection. As illustrated in Figure 3, both Ig-HA and Ig-HA-mPEG were taken up by the various organs to the same extent but the amounts of Ig-HA-mPEG retained in the organs were higher than those of native Ig-HA. It should be noted that in lungs, while Ig-HA-mPEG was still present by 168 hours, the native Ig-HA was cleared by 72 hours. Overall, these results indicate that pegylated Ig-HA molecules persist longer than the native Ig-HA in the blood, and higher amounts of the pegylated Ig-HA were retained in organs with lymphoid tissues.

The cellular immune response to HA110-120 peptide expressed by pegylated Ig-HA chimera.

In vitro activation of HA110-120 specific T cell hybridoma. To assess the effect of mPEG derivatization on the immunogenicity of Ig-HA, we studied in vitro T cell activation by native Ig-HA and Ig-HA-mPEG. This was carried out using HA110-120 specific T hybridoma cells (LD1-24 cells) and measurement of IL-3 release by these cells subsequent to activation by the various antigens. Figure 4 shows that Ig-HA-mPEG activated the specific T cells to the same extent as native Ig-HA. This indicates that mPEG derivatization of the chimeric Ig did not affect the delivery and subsequent presentation of the HA100-120 peptide to the T

cells. No activation was observed with pegylated Ig-NP, a molecule carrying a CTL epitope from NP of PR8 influenza A virus instead of the HA110-120 helper epitope.

In vivo priming of HA110-120 specific T cells. Immunization of BALB/c mice with Ig-HA in CFA primed animals results in a specific proliferative response (Zaghouani, et al., 1993, Science 259:224). In this study, we compared the immunogenicity of native and pegylated Ig-HA administered in either saline or CFA (Figure 5). Lymph node cells from BALB/c mice were harvested ten days after immunization and stimulated in vitro for 5 days with various antigens. Immunization with native Ig-HA in CFA primed T cells resulted in an in vitro proliferative response upon incubation with Ig-HA, UV-inactivated PR8 virus and HA110-120 peptide but not Ig-NP or NP 147-161 peptide (Figure 5b). Similar responses were obtained when the mice were primed with Ig-HA-mPEG in CFA (Figure 5d).

More strikingly, a strong and specific proliferative response was obtained when the mice were primed with Ig-HA-mPEG in saline (Figure 5c). No response was detected when the mice were immunized with native Ig-HA in saline (Figure 5a). This strongly suggests that the requirement for adjuvant was obviated when Ig-HA was derivatized with mPEG 5,000.

Effect Of Ig-V3C derivatization with mPEG on the antibody response to V_3C peptide and to Ig-xenogeneic determinants

The kinetics of the antibody response elicited by native and pegylated Ig-V_3C was studied in BALB/c mice following different immunization schedules (see the legend for Figure 6). As mentioned above, Ig-V_3C is a chimeric molecule made up of murine V_H gene expressing the consensus V_3C peptide, murine V_k gene and

human γ1 and κ constant region genes. Therefore, we measured by RIA the antibody response against the V_3C epitope as well as against isotypic determinants of human constant regions.

The study showed a sharp increase of the antibodies to V_3C peptide after immunization with Ig-V_3C-mPEG in CFA followed by a boost in IFA (Figure 6a). More importantly, immunization of animals with Ig-V_3C or Ig-V_3C-mPEG in saline induced a V_3C specific antibody response (Figures 6b, 6c, and 6d). This response was slightly higher in animals immunized with Ig-V_3C-mPEG. The antibody response was stronger when the animals were immunized 3 times in saline (Figure 6b versus Figures 6c and 6d).

The animals immunized with native chimeric Ig in CFA showed high antibody response to isotypic determinants of human constant regions (Figure 6e). However, while immunization in saline with native Ig-V_3C also induced a strong anti-human antibody response, immunization with Ig-V_3C-mPEG in saline elicited no significant response (Figures 6f, 6g and 6h).

7. EXAMPLE: PURIFICATION OF PEGYLATED IMMUNOGLOBULIN

During pegylation of a protein, various degrees of derivatization may occur as a consequence of the micro heterogeneity of the protein, the distribution of both the number and the position of attachment of PEG units, the inherent polydispersity of PEG polymers, and microenvironmental conditions of the reaction. In addition, deoxy PEG that can crosslink proteins may be present in commercial preparations as a result of hydrolysis of the ethylene oxide monomers during the polymerization process (Selisko et al., 1993, J. Chromatogr. 641: 71-79; Harris et al., 1985, in "Partitioning in Aqueous Two-Phase Systems", D.E. Brooks and D. Fisher (Editors), Academic Press,

Orlando, Fl., p.593). Thus, protein-mPEG preparations may contain species of highly pegylated and/or cross-linked proteins in addition to the residual adducts. Purification of homogeneous populations of conjugates is therefore required prior to their use in biological systems. Purification of protein-PEG conjugates has generally consisted of a single step chromatographic removal of free PEG polymers (Kitamura, et al., 1991 Cancer Res., 51: 4310-43115; Cunningham-R.A.et al., 1992, J. Immunol. Methods, 152: 177-190; Snider et al., 1992, J. Chromatogr. 599: 141-155; Jackson, et al., 1987, Anal-Biochem. 165:114-127). Herein, we describe a two step procedure able to remove completely the residual adducts and to isolate homogeneous populations of mPEG derivatized immunoglobulins.

7.1. MATERIALS AND METHODS

Materials. AIgs (Ig-HA and Ig-V_3C) were generated in our laboratory as described above. PhastSystem electrophoresis apparatus, PhastGels 4-15% gradient of polyacrylamide and cyanogen bromide activated Sepharose CL-4B were purchased from Pharmacia, LKB. AcA44 Ultrogel and dialysis bags of 75,000 MWCO were from Spectrum, Co. Ultra concentrators of 100,000 MWCO (CENTREX UF-2, 2ml volume) were from Schleicher & Schuell. Rat hybridoma cells secreting monoclonal anti-murine k chain antibodies and HP6053 mouse hybridoma producing anti-human k chain antibodies were from ATCC. The Q300 HPLC column (250 x 4.6 mm, 300 micron particle size) was from Rainin. Nessler reagent and 2,4,6-trichloro-s-triazine activated monomethoxypolyethylene glycol MW 5,000 (mPEG 5,000) were from Sigma. The agarose gels (Titan Gel High-Resolution Protein kit) were from Helena Laboratories.

Purification of AIgs. IgHA and Ig-V_3C were affinity purified on a rat anti-murine k chain and

mouse anti-human k chain antibody-Sepharose columns, respectively. Affinity purified AIgs were equilibrated in 0.1M sodium tetraborate, pH 9.6 and concentrated to 1 mg/ml by ultracentrifugation using tubes of 100,000 MWCO.

Derivatization of AIgs with mPEG. 10 mg of each AIg were derivatized with 2,4,6-trichloro-s-triazine activated mPEG 5,000 as described in Jackson, et al., 1987, Anal-Biochem. 165:114-127. Briefly, a 50 times molar excess of mPEG was added to 10 mg AIgs in 10 ml of 0.1M tetraborate buffer, pH 9.6. The mixture was stirred vigorously for four hours at room temperature. The conjugate preparations were concentrated to 1.5 ml in tubes of 100,000 MWCO and further purified.

Chromatographic purification of AIg-mPEG conjugates. AIg-mPEG preparations were applied to a AcA44 Ultrogel filtration column (80 x 1.6cm) equilibrated with 0.1M NH_4HCO_3, pH 8.5 and flow rate of 0.4 ml/min. Fractions were collected at 4 min interval, dried by speed vacuum centrifugation and resuspended in 0.5 ml of 5mM sodium-acetate, pH 5. Each fraction was then analyzed for protein content by Biuret micro assay, and for the presence of free hydrolyzed mPEG by Nessler's reagent as described in Wilkinson et al., 1987, Immunol.Lett. 15: 17-22. Fractions of the conjugates free of hydrolyzed mPEG were then rechromatographed on a Q300 anion-exchange HPLC column equilibrated with 5 mM sodium acetate, pH 5 using a 45 minutes linear gradient from 5 to 500 mM sodium acetate, pH 5, and flow rate of 0.5 ml/min. Fractions from the Q300 column were dialyzed in Spectrapor bags with 75,000 MWCO against PBS and concentrated in CENTREX UF-2 tubes.

Electrophoretic analyses of AIg-mPEG conjugates. Homogeneity of AIg-mPEG conjugates were analyzed by SDS-PAGE and residual mPEG was detected by

electrophoresis on agarose gels. The SDS-PAGE was performed under non-reducing conditions as described by Laemmli (Laemmli, 1970, Nature (London), 227: 680-685). Briefly, Samples (5 μg) were resuspended in 5 μl of sample buffer (0.1% SDS, 0.1M Tris-HCl 6M urea, pH 8.5) and incubated at 37°C for 2 hours. Samples were run on 4-15% gradient PhastGel (Pharmacia) at 250 Volts and 5 Watts for 45 minutes at 15°C. The gel was then fixed and silver stained. Electrophoresis on agarose Titan gels was carried out according to the manufacture's instructions. Samples (10 μg in 5 μl barbital buffer) were run at 250 Volts for 30 minutes on duplicate gels. One gel was stained with a mixture of 0.1% Coomassie Blue R-250 and 1% Ponceau S, and the second gel was immersed in 20% trichloroacetic acid (TCA) in water for 30 seconds and immediately photo-scanned.

Fluorescamine assay. Graded amounts (1, 0.5 and 025 μg) of AIg-mPEG or AIgs in 25 μl of PBS were mixed with equal volume of fresh fluorescamine in acetone (150 g/ml) and incubated for 5 min at room temperature. Samples were then brought to 1 ml with PBS and absorbency was measured at 475 nm emission versus 390 nm excitation. The percent of pegylated primary amines was estimated according to the following formula $1-(OD_{475}AIgs\text{-}mPEG/OD_{475}AIgs)x100$ (Neville, et al., 1991, presented at the American Association of Pharmaceutical Scientists', Sixth Annual Meeting, Washington, DC, Nov. 17-21, 1991). The improved resolution obtained with ammonium bicarbonate may be related to better neutralization of the active charges on the gel matrix and therefore lower interactions between free mPEG polymer and the matrix.

7.2. RESULTS

Removal of other residual adducts from the AIg-mPEG conjugates. Because no one step procedure for

the separation of protein-mPEG conjugates from residual adducts has been described, we chose a method that includes a first step removal of hydrolyzed mPEG and a subsequent step that removes other residual adducts such as underivatized or highly derivatized AIgs. Since the size exclusion limit of the AcA44 Ultrogel column is 140 kD, hydrolyzed mPEG can be removed but the other residual adducts can not. The removal of excess PEG is required because it could interfere with the isolation of the conjugates from the other residual adducts in the subsequent anion exchange chromatography step. Preliminary experiments performed on anion-exchange HPLC columns as a single step of purification, showed poor resolution and low yields even if minimal amounts of sample were applied on the column. In fact, the conjugates showed a broad elution profile as a result of the presence of excess of free polymers which interfere strongly with the binding of conjugates to the anion-exchange matrix. Poor resolution of pegylated proteins was also described when conjugates were separated by charge-reversal capillary zone electrophoresis without previous removal of free PEG (Snider et al., 1992, J. Chromatogr. 599: 141-155; McGoff, et al., 1988, Chem.Pharm.Bull. 36: 3079). It may be important to remove free polymer from the conjugate preparation in order to obtain good resolution in different separating media.

To select a homogeneous population of conjugates with 6-8% degree of pegylation, we rechromatographed the conjugates on anion-exchange HPLC column with optimized conditions as described in the experimental section. Figure 7 shows the elution profiles of Ig-HA-mPEG (Figure 7A) and free IG-HA (Figure 7b). In the case of Ig-HA-mPEG, 3 major peaks were eluted from the column and labeled 1, 2, and 3 (Figure 7a). Peak 3 represents free Ig-HA since it elutes at the same salt

concentration as the unconjugated control Ig-HA. The material of peak 1 may represent highly pegylated AIgs that could not bind to the matrix. Peak 2 contained mildly pegylated Ig-HA as revealed by SDS-PAGE analysis (see below).

Analysis of the homogeneity of AIg-mPEG conjugates. The purity of AIgs-mPEG preparations was further analyzed for the presence of traces of free mPEG and residual adducts performed on agarose and polyacrylamide gel electrophoresis, respectively. Although purification of AIgs-mPEG conjugates followed one step purification by size exclusion chromatography, small amounts of free mPEG that could not be detected by Nessler's test may be present in the conjugate preparations. To trace small amounts of free PEG that could interfere with the immunogenicity of the AIgs-mPEG we developed an original, sensitive electrophoretic technique able to detect μM of free mPEG. Based on the observation that mPEG migrate on agarose to the cathode and can be visualized by TCA precipitation but not by protein dyes, we attempted to trace free mPEG in our preparation by electrophoresis on a Titan gel HRT kit followed by TCA precipitation. As can be seen in Figure 8, left panel, an Ig-HA-mPEG preparation obtained from the second chromatographic purification (lane 1) like unpegylated Ig-HA preparation (lane 2), did not show detectable amounts of free hydrolyzed mPEG while the preparation collected from the first chromatographic purification contained residual free PEG (lane 4). The amount of free residual mPEG, if any, in the final Ig-HA-mPEG preparation (lane 1) should be lower than 4×10^{-4} M. Calibration experiments indicated that as little as 4×10^{-4}M of mPEG 5,000 can be detected using this technique (lane 3).

When a duplicate gel was stained with Coomassie/Ponceau (Fig. 8, right panel), only Ig-HA and Ig-HA-mPEG conjugates were revealed (lanes 1, 2, respectively) but not free hydrolyzed mPEG (lane 3). Interestingly, pegylated Ig-HA showed better staining with Ponceau S than unpegylated Ig-HA. The differential staining may be attributed to different ability of the two dyes to access their specific sites on the native protein versus the pegylated one. Using this assay, we also were able to trace free mPEG polymers in preparations of chicken egg ovalbumin-mPEG, BSA-mPEG and bovine gamma globulin-mPEG conjugates.

Although small amounts of PEG polymers can be detected by sensitive techniques such as Childs' assay (Childs, 1975, Microchem. J. 20: 190-192) or Veronese's test (Schiavon, et al., 1990, M. Farmaco., 45(6): 791-795), our electrophoretic technique is able to distinguish free PEG from PEG attached to proteins.

Furthermore we performed SDS-PAGE analysis to investigate the homogeneity of AIgs-mPEG preparations. This was carried out on 4-15% gradient gels (PhastGels, Pharmacia) under non-reducing conditions. The results illustrated in Figure 9 show that the final Ig-HA-mPEG and Ig-V_3C-mPEG preparation (peak 2 in Figure 7) were purified to homogeneity (lanes 2 and 5 respectively). These preparations do not contain detectable amounts of unpegylated AIgs because no comigration with free Ig-HA (lane 1) or Ig-V_3C (lane 4) was observed. They do not contain highly pegylated AIgs either because no material comigrated with samples representing heavily pegylated AIgs (from peak 1 in Figure 7). In summary, we were able to select a homogeneous population of AIg-mPEG with 6-8% degree of pegylated as indicated by these electrophoretic analyses.

The pegylated Ig-HA, showed a long half life and induced strong T cell activation in mice *in vivo*.

8. EXAMPLE: ENZYMATIC DERIVATIZATION OF IMMUNOGLOBULINS WITH PEG

As set forth in the preceding sections, immunoglobulins have been found to exhibit a remarkable increase in half-life when 6 - 8% of the primary amines are chemically conjugated to mPEG 5,000. However, whereas such mild pegylation may preserve the immunogenicity of hidden determinants on immunoglobulins, higher degrees of pegylation may produce undesirable tolerogenic and/or suppressogenic effects for foreign epitopes present on immunoglobulins used for immunization purposes. Tolerization of an immunogenic epitope may occur as a consequence of PEG attachment to the particular epitope. Similarly, pegylation of a CDR may destroy the functional activity of the CDR. In order to avoid such undesirable effects, PEG has been targeted to carbohydrate moieties found on immunoglobulin molecules in order to increase the half-life of the immunoglobulins while preserving their biological activity.

8.1 MATERIALS AND METHODS

Immunoglobulins. Human IgA from colostrum (Sigma) and a rat IgG2b anti-mouse k chain monoclonal antibody were used for glycopegylation in this study. Ig-HA is a BALB/c IgG2b comprising, in the CDR3 loop, a T cell epitope (HA110-120) from the hemagglutinin (HA) of PR8 influenza A virus. IgW is a BALB/c IgG2b comprising the unmutated V_H region of the anti-arsonate 91A3 mAb. Ig-TB is an Ig-HA chimera in which a HA150-163 B cell epitope was genetically engineered into the CDR2 loop. To remove traces of IgG in the preparation of IgA, size exclusion chromatography on Superose-6 column was performed as described below. Rat anti-mouse K chain mAb was purified from cell culture super-

natant by affinity chromatography on a mouse anti-rat (MAR 18.5) sepharose column. The genetically engineered chimeras were purified from cell culture supernatants by affinity chromatography on rat anti-mouse k chain-sepharose column.

Enzymatic Synthesis of Glycopegylated Igs. Human asialo-IgA colostrum (1mg) in 1 ml of borate buffer 0.1M, pH 9.3 was mixed with galactose oxidase (GAO, Sigma), 25 U/mg of Ig, glycine-PEG 3,500 derivative (Sharewater, U.S.A) at 80 fold molar excess relative to the amount of immunoglobulin present, and 80mM of pyridine borane (PB, Aldrich). The reaction mixture was incubated overnight at 37°C, with continuous stirring. Rat anti-mouse k chain mAb (1mg) was first desialylated with a mixture of neuraminidases (50 mU, 1:1) from Arthrobacter ureafaciens (90.8 U/mg protein), and Clostridium perfringens (4.8 U/mg protein, Calbiochem) by incubating the reaction mixture overnight at 37°C with 1ml of phosphate buffer, pH 6 containing 5 mM of $CaCl_2$. The kinetics of the desialylation reaction may be monitored by determining free NANA released in solution, as described in Warren, 1959, J. Biol. Chem. 234:1971. Free N-acetyl neuraminic N-acetyl acid (sialic acid, NANA) was then removed by dialysis against 0.1M borate buffer, pH 9.3 and the glycopegylation reaction was continued as for the IgA preparation. A diagram of the reaction is set forth in Figure 11. The glycopegylated Igs (Ig-Gal-PEG) were rendered free of unreacted Gly-PEG by FPLC size exclusion chromatography and then further analyzed.

Purification of Glycopegylated Igs. Ig-Gal-PEG conjugates were concentrated by speed vacuum centrifugation and applied to a Superose-6 HR10/30 column (Pharmacia, LKB) equilibrated in PBS at a flow rate of 0.2 ml/min. Fractions were collected every minute and

the tubes containing the conjugates were pooled and tested for protein concentration by Biuret micro-assay and for the presence of free PEG using methods set forth in the preceding sections. The chromatographic profiles were monitored at the maximum absorbance for PEG (254nm). Ig-Gal-PEG conjugates were concentrated and used for further investigations.

8.2. RESULTS

Electrophoretic Analyses of Ig-Gal-PEG Conjugates. The purity and homogeneity of Ig-Gal-PEG conjugates were analyzed by SDS-PAGE under non-reducing or reducing conditions using methods set forth in the preceding sections. For SDS-PAGE analysis, samples (5 μg) were resuspended in 5 μl of sample buffer (0.1% SDS, 0.1M Tris-HCl 6M urea, pH 8.5) with or without 2-mercaptoethanol (5%) and incubated at 37°C for 2 hours. Samples were run on 4-20% gradient gels (BioRad) at 100 Volts for 60 minutes. A representative resulting gel was then fixed and Coomassie stained (Figure 12).

To identify charge modifications of the immunoglobulins after glycopegylation, the Ig-Gal-PEG conjugates were analyzed in comparison with native Igs (20μg in 10μl) by isoelectric focusing. Electrophoresis was carried out in 5% polyacrylamide gels (PAG plate, pH 3-10 Pharmacia, LKB) for 90 min at 15°C and 1500 Volts, 50 Watts and 30 mAmps. Gels were stained with Coomassie and photographed (Figure 13). The pI values were determined on a pI standard curve constructed with a broad pI range standards (Pharmacia, LKB). Both SDS-PAGE and IEF electrophoregrams were scanned in a Phosphoimage densitometer and the percent of the separated bands was calculated using "Image-Quant" software. Traces of free Gly-PEG, which may remain in the preparation of Ig-Gal-PEG conjugates after purification by size exclusion chromatography, were monitored by electrophoresis on agarose Titan gels

(Helena Labs, TX, Beaumont) using methods set forth in the preceding sections. Briefly, samples (10 μg in 5 μl barbital buffer) were run at 250 Volts for 30 minutes on duplicate gels. One gel was stained with 0.1% Coomassie Blue R-250 and the second gel was immersed in 20% trichloroacetic acid (TCA) in water for 30 seconds for detection of unreacted Gly-PEG (Figure 14).

Estimation of GAO Reactive Sites. To estimate the number of galactose sites on carbohydrate moieties of Igs that can potentially participate in the coupling reaction with Gly-PEG, we examined the enzymatic oxidation of Igs using a tilidine-horseradish peroxidase coupled assay system. Briefly, 1ml of a reaction mixture containing either 0.5 mg immunoglobulin (e.g., human IgA, rat anti-mouse k chain mAb, mouse IgG1 B43.13 mAb), or graded amounts of D-galactose (0.019 to 5 μg) was mixed with 30 U horseradish peroxidase, 0.5 mg tilidine and 2.5 U GAO in 0.1M sodium phosphate at pH 7. The mixture was incubated for 18 h at 37°C and the absorbance was determined at 425 nm. A calibration curve using D-galactose was used to determine the relative "galactose equivalents" on native Igs. Mouse IgG1 B43.13 mAb has been used in reaction as a known Ig containing 6.3 nM of galactose residues which corresponds to 4 Gals but showing only 2.1 nM reactive galactose residues (1 residue). The results of these studies were that the potential galactose sites available for coupling to PEG were four for the rat IgG2b anti-mouse k chain MAB and eight for human IgA from colostrum.

Estimation of GAO Activity in the Presence of Gly-PEG. To determine whether or not Gly-PEG polymer may interfere with the enzymatic oxidation of Gals during the conjugation reaction, we have tested the enzymatic activity of GAO in the presence of graded

amounts of Gly-PEG and using D-Galactose as the substrate for the enzymatic reaction. A reaction mixture made of GAO (2.5 U), D-galactose (1 μg), 30 U horseradish peroxidase, 0.5 mg tilidine and 80mM of pyridine borane (PB) in 0.1M sodium phosphate at pH 7 was incubated for 18 h at 37°C and the absorbance was determined at 425 nm. The same reaction mixture was tested in the presence of graded amounts of Gly-PEG 3,500 corresponding to similar GAO/Gly-PEG molar ratios that may be used in the coupling reaction of Igs with Gly-PEG 3,500 (Figure 16). The results of these studies were that slightly increased GAO activity was observed when PEG was co-incubated in the reaction mixture.

Various publications are cited herein which are hereby incorporated by reference in their entirety.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK

(ii) TITLE OF THE INVENTION: PEGYLATED MODIFIED PROTEINS

(iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Brumbaugh, Graves, Donohue & Raymond
(B) STREET: 30 Rockefeller Plaza
(C) CITY: New York
(D) STATE: NY
(E) COUNTRY: USA
(F) ZIP: 10112-0228

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT US APPLICATION DATA:
(A) APPLICATION NUMBER: 08/477,421
(B) FILING DATE: 7-JUNE-1995
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Clark, Richard S
(B) REGISTRATION NUMBER: 26,154
(C) REFERENCE/DOCKET NUMBER: 29889-165/29528

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-408-2558
(B) TELEFAX: 212-765-2519
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency Virus Type 1

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 301...319
(C) OTHER INFORMATION: Envelope Protein gp120

(v) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Arg Lys Ser Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Gly
1 5 10 15
Glu Ile Ile

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Influenza Virus

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) OTHER INFORMATION: HA1 hemagglutinin protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Trp Leu Thr Lys Lys Gly Asp Ser Tyr Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Influenza Virus

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) OTHER INFORMATION: H3 protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Trp Leu Thr Lys Ser Gly Ser Thr Tyr Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Influenza Virus

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) OTHER INFORMATION: H2 protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Trp Leu Thr Lys Glu Gly Ser Asp Tyr Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Measles Virus

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 404...414
(C) OTHER INFORMATION: F protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ile Asn Gln Asp Pro Asp Lys Ile Leu Thr Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Foot and Mouth Disease Virus

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 141...160
(C) OTHER INFORMATION: VP1 protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Asn Ser Ala Pro Asn Leu Arg Gly Asp Leu Gln Lys Val Ala Arg
1 5 10 15
Thr Leu Pro

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Influenza PR8A Virus

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 110...120
(C) OTHER INFORMATION: Hemagglutinin Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Phe Glu Arg Phe Glu Ile Phe Pro Lys Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM:

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) OTHER INFORMATION: Tetanus Toxoid Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asn Ser Val Asp Asp Ala Leu Ile Asn Ser Thr Lys Ile Tyr Ser Tyr
1 5 10 15
Phe Pro Ser Val
20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM:

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) OTHER INFORMATION: Tetanus Toxoid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Pro Glu Ile Asn Gly Lys Ala Ile His Leu Val Asn Asn Glu Ser Ser
1 5 10 15
Glu

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM:

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 88...103
(C) OTHER INFORMATION: Cytochrome C Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Asn Glu Arg Ala Asp Leu Ile Ala Tyr Leu Gln Ala Thr Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Mycobacteria

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 350...369
(C) OTHER INFORMATION: Heat Shock Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Asp Gln Val His Phe Gln Pro Leu Pro Pro Ala Val Val Lys Leu Ser
1 5 10 15
Asp Ala Leu Ile
20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Hen

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 48...61
(C) OTHER INFORMATION: Egg White Lysozyme

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Asp Gly Ser Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Streptococcus A

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 308...319
(C) OTHER INFORMATION: M Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gln Val Glu Lys Ala Leu Glu Glu Ala Asn Ser Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Staphylococcus sp.

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 81...100
(C) OTHER INFORMATION: Nuclease Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Thr Asp Lys Tyr Gly Arg Gly Leu Ala Tyr Ile Tyr Ala Asp Gly
1 5 10 15
Lys Met Val Asn
20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:

(A) ORGANISM:

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Ala Ala Leu
1

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ORIGINAL SOURCE:

(A) ORGANISM: Influenza PR8A Virus

(iv) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 147...161
(C) OTHER INFORMATION: NP Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Tyr Gln Arg Thr Arg Ala Leu Val Arg Thr Gly Met Asp Pro
1 5 10 15

WHAT IS CLAIMED IS:

1. A method of preparing a substantially purified immunoglobulin molecule covalently conjugated to a polyethylene glycol molecule, the immunoglobulin molecule containing an immunogenic heterologous peptide selected from the group consisting of a T cell and a B cell epitope, the method comprising the steps of:

(i) enzymatically oxidizing a carbohydrate residue of the immunoglobulin molecule;

(ii) reacting the oxidized carbohydrate residue with an activated form of polyethylene glycol;

(iii) stabilizing the reaction product of step (ii) by reaction with a reducing agent;

(iv) removing unreacted polyethylene glycol from the products of the conjugation reaction of steps (i-iii); and

(v) removing residual adducts from the products of step (iv) by anion exchange chromatography.

2. The method of claim 1, wherein the activated form of polyethylene glycol is an amino acid derivative of polyethylene glycol.

3. The method of claim 2, wherein the activated form of polyethylene glycol is glycine-polyethylene glycol.

4. The method of claim 3, wherein the glycine polyethylene glycol is glycine-polyethylene glycol 3,500.

5. The method of claim 1, wherein the unreacted polyethylene glycol is removed from the products of the conjugation reaction by size exclusion chromatography.

6. The method of claim 1, wherein the residual adducts are removed by anion exchange chromatography using high pressure liquid chromatography.

7. The method of claim 6, wherein residual adducts are removed by anion exchange chromatography using high pressure liquid chromatography, wherein a Q300 anion exchange column is used.

8. A composition comprising a substantially purified immunoglobulin molecule prepared using the method of claim 1.

9. A composition comprising a substantially purified immunoglobulin molecule prepared using the method of claim 2.

10. A composition comprising a substantially purified immunoglobulin molecule prepared using the method of claim 3.

11. A composition comprising a substantially purified immunoglobulin molecule prepared using the method of claim 4.

12. A substantially purified biologically active immunoglobulin molecule containing an immunogenic heterologous peptide selected from the group consisting of a T cell and a B cell epitope, wherein the immunoglobulin molecule is covalently conjugated to an effective amount of polyethylene glycol for increasing the half-life of the immunoglobulin molecule without substantially decreasing the biological activity of the immunoglobulin molecule or the immunogenicity of the heterologous peptide, wherein the polyethylene glycol is conjugated to the immunoglobulin via a carbohydrate residue.

13. A method of preparing a non-immunoglobulin molecule covalently conjugated to a polyethylene glycol molecule, wherein the non-immunoglobulin molecule is a protein comprising at least one carbohydrate residue, the method comprising the steps of:

(i) enzymatically oxidizing a carbohydrate residue of the protein;

(ii) reacting the oxidized carbohydrate residue with an activated form of polyethylene glycol;

(iii) stabilizing the reaction product of step (ii) by reaction with a reducing agent;

(iv) removing unreacted protein from the products of the conjugation reaction of steps (i-iii); and

(v) removing residual adducts from the products of step (iv) by anion exchange chromatography.

14. The method of claim 13, wherein the activated form of polyethylene glycol is an amino acid derivative of polyethylene glycol.

15. The method of claim 14, wherein the activated form of polyethylene glycol is glycine-polyethylene glycol.

16. A protein conjugated to polyethylene glycol by the method of claim 13.

17. A protein conjugated to polyethylene glycol by the method of claim 14.

18. A protein conjugated to polyethylene glycol by the method of claim 15.

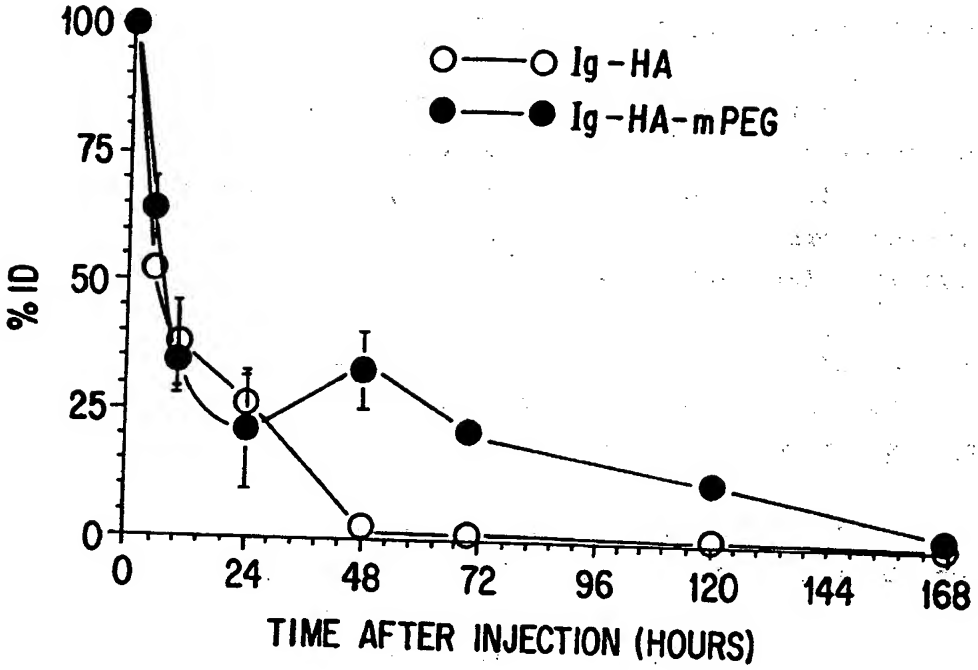
100
75
50
25
0
% ID
0
24
48
72
96
120
144
168
TIME AFTER INJECTION (HOURS)
Ig-HA
Ig-HA-mPEG

FIG. 1

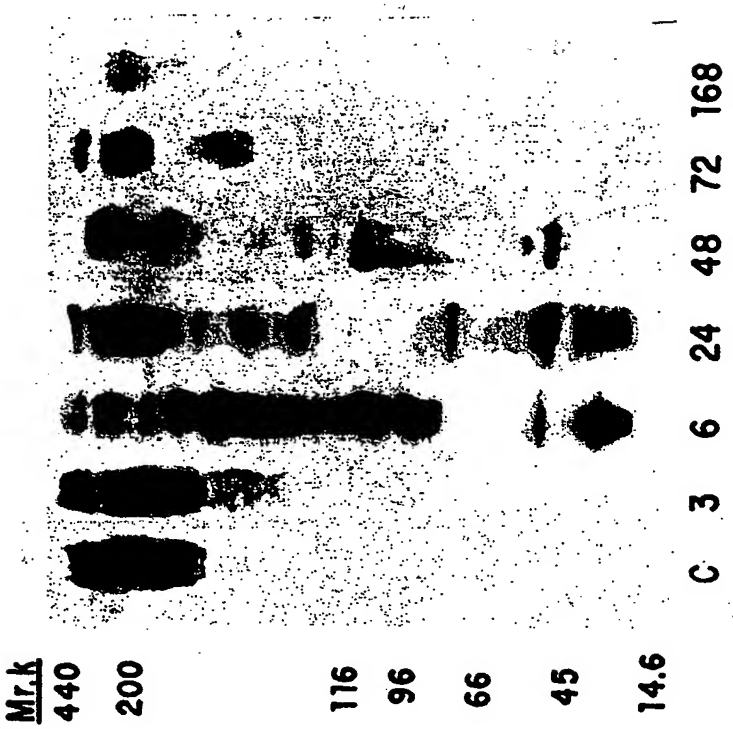
Mr.k
440
200
116
96
66
45
14.6
C 3 6 24 48 72 168

FIG. 2B

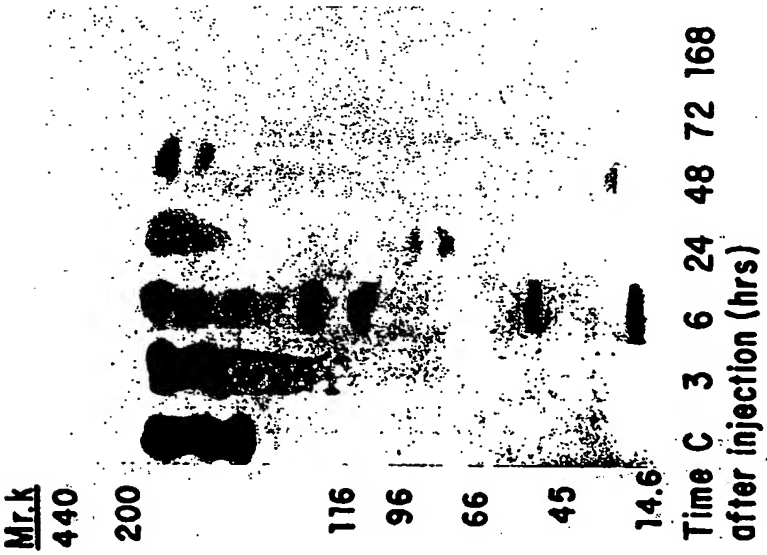
Mr.k
440
200
116
96
66
45
14.6
Time C 3 6 24 48 72 168
after injection (hrs)

FIG. 2A

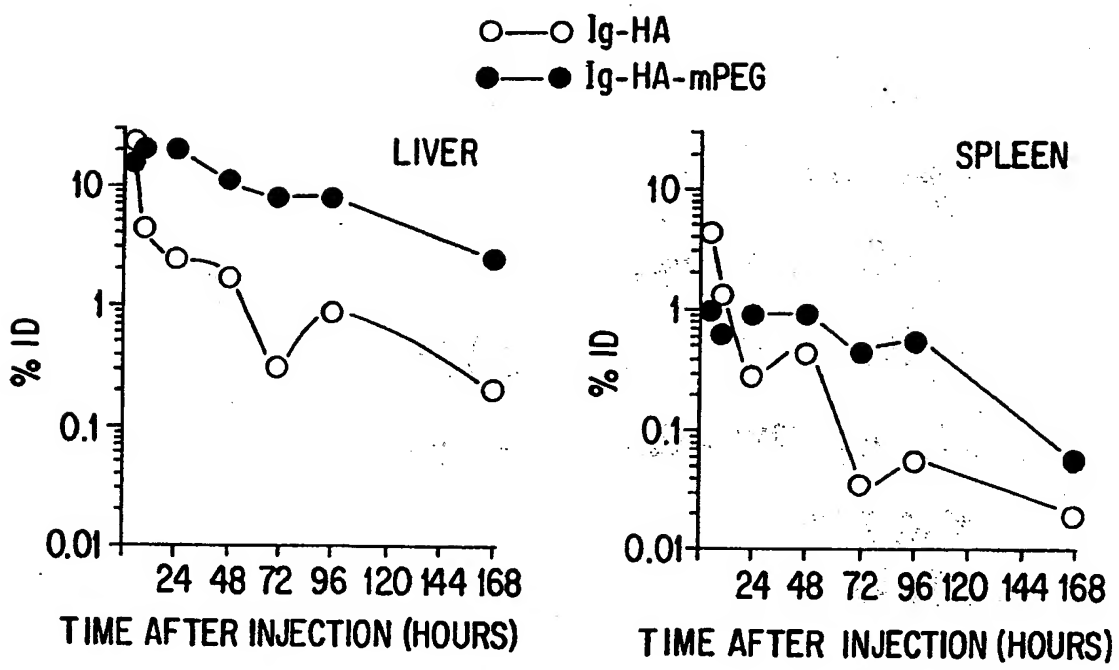

FIG. 3A

FIG. 3B

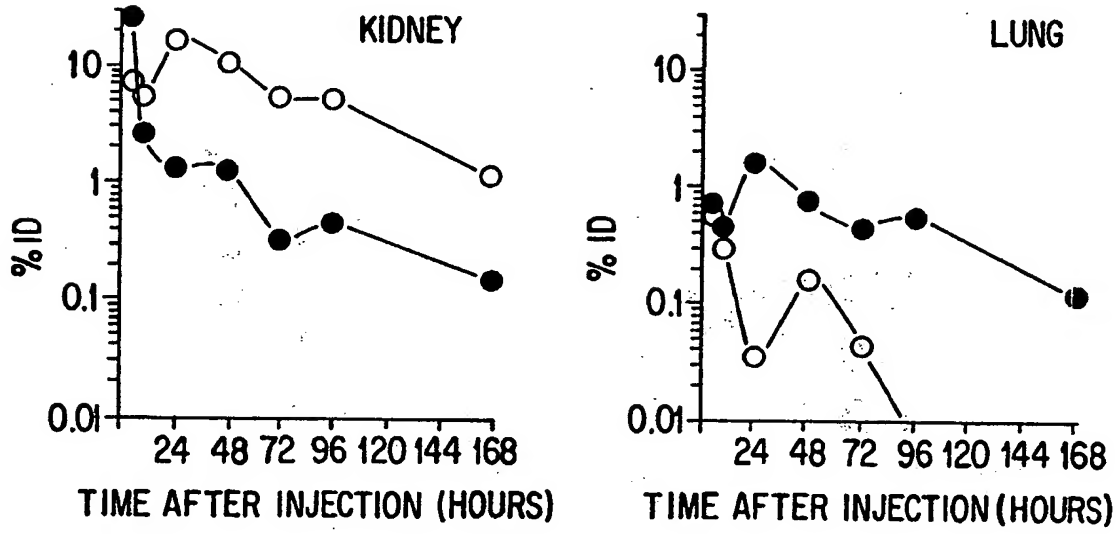

FIG. 3C

FIG. 3D

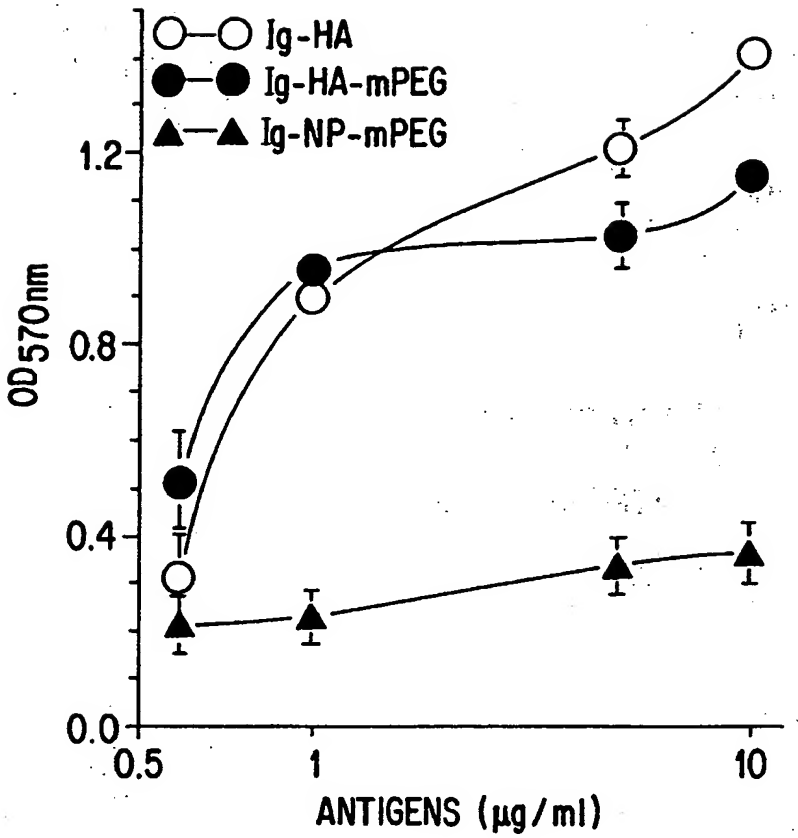
Ig-HA
Ig-HA-mPEG
Ig-NP-mPEG
1.2
0.8
0.4
0.0
OD570nm
0.5
1
10
ANTIGENS (μg/ml)

FIG. 4

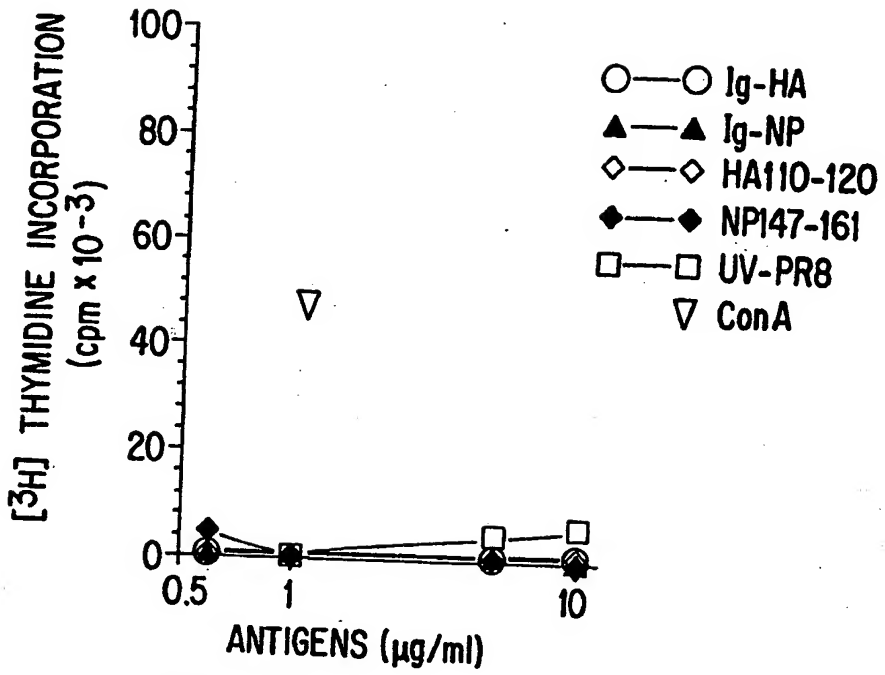
[3H] THYMIDINE INCORPORATION (cpm x 10-3)
100
80
60
40
20
0
0.5
1
10
ANTIGENS (µg/ml)
Ig-HA
Ig-NP
HA110-120
NP147-161
UV-PR8
ConA

FIG. 5A

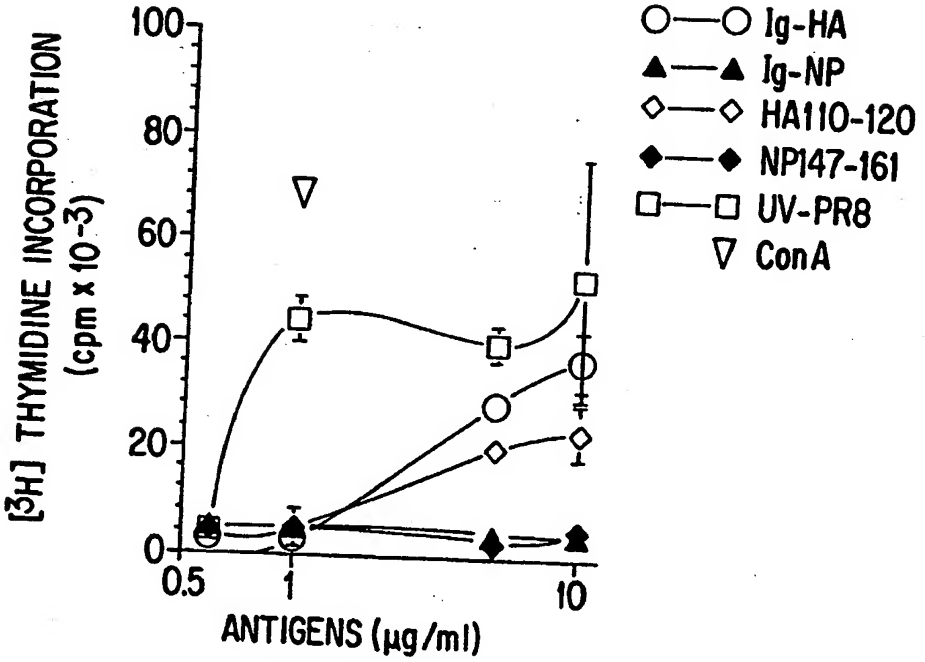
[3H] THYMIDINE INCORPORATION (cpm x 10-3)
100
80
60
40
20
0
0.5
1
10
ANTIGENS (µg/ml)
Ig-HA
Ig-NP
HA110-120
NP147-161
UV-PR8
ConA

FIG. 5B

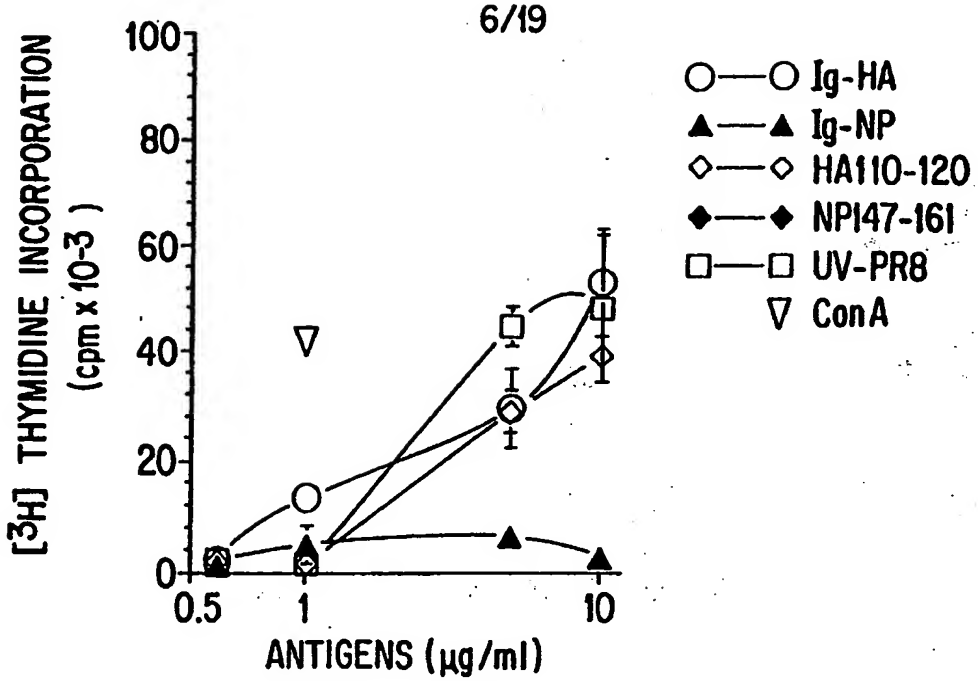
[3H] THYMIDINE INCORPORATION (cpm x 10-3)
100
80
60
40
20
0
0.5
1
10
ANTIGENS (µg/ml)
Ig-HA
Ig-NP
HA110-120
NP147-161
UV-PR8
ConA

FIG. 5C

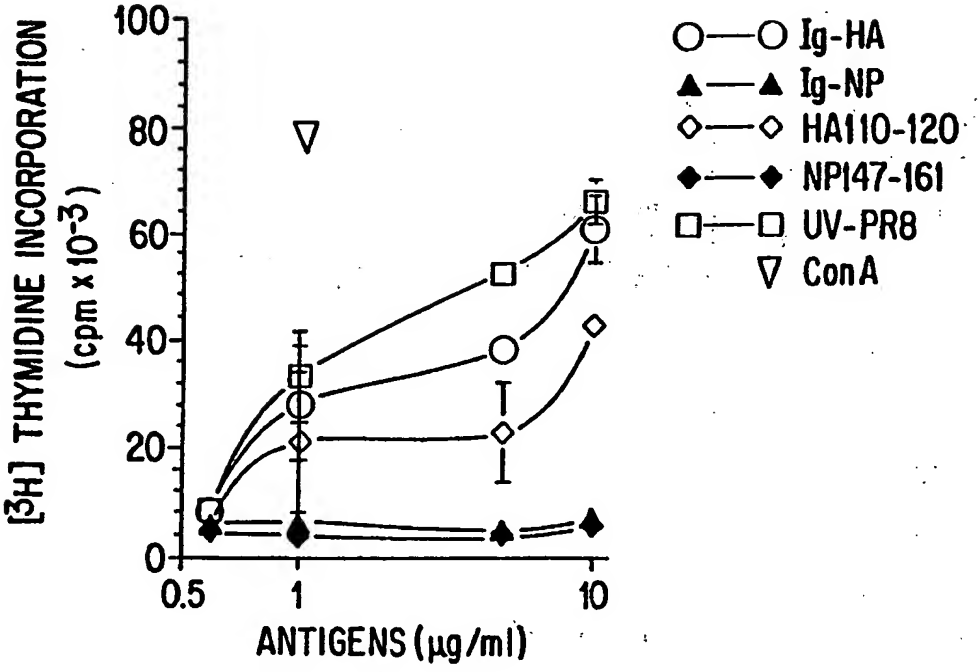
[3H] THYMIDINE INCORPORATION (cpm x 10-3)
100
80
60
40
20
0
0.5
1
10
ANTIGENS (µg/ml)
Ig-HA
Ig-NP
HA110-120
NP147-161
UV-PR8
ConA

FIG. 5D

o—o Ig-V3C

●—● Ig-V3C-mPEG

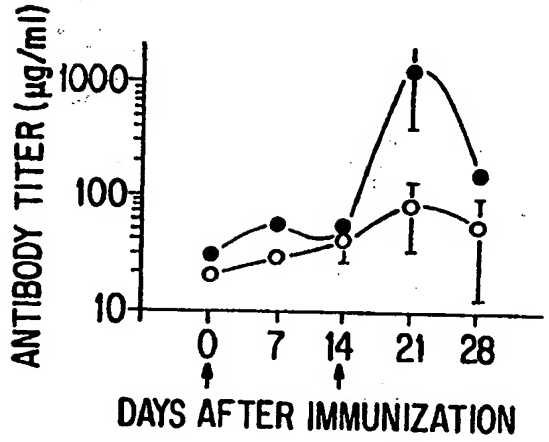

FIG. 6A

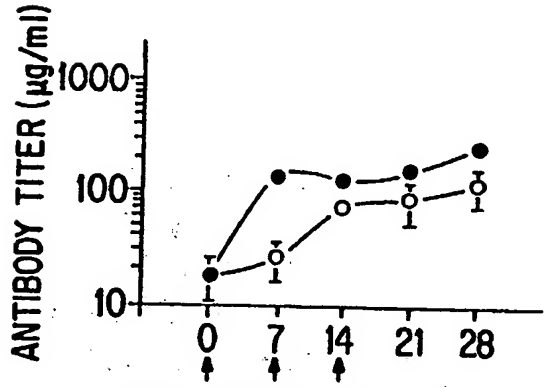

FIG. 6B

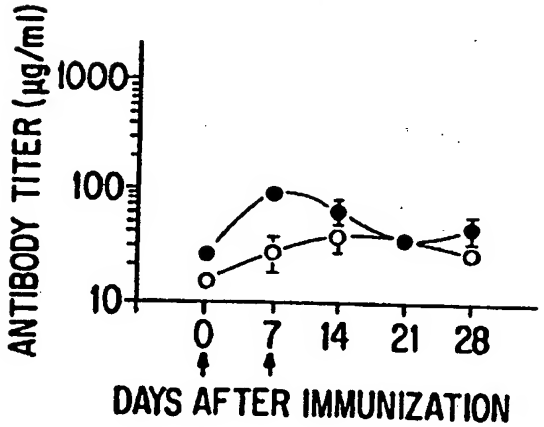

FIG. 6C

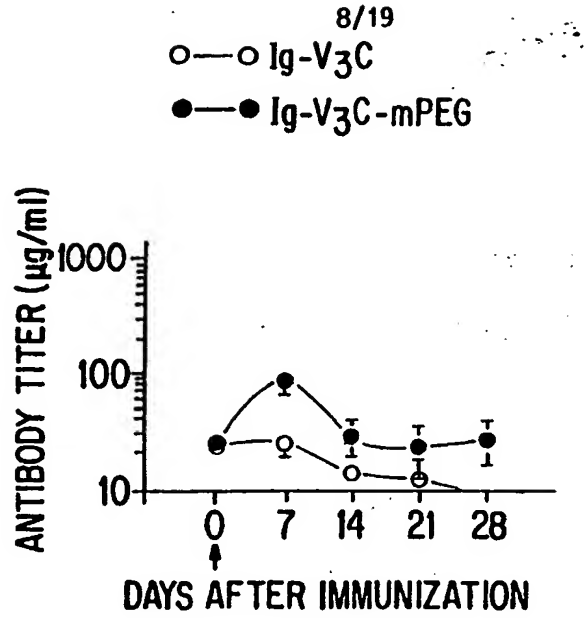

FIG. 6D

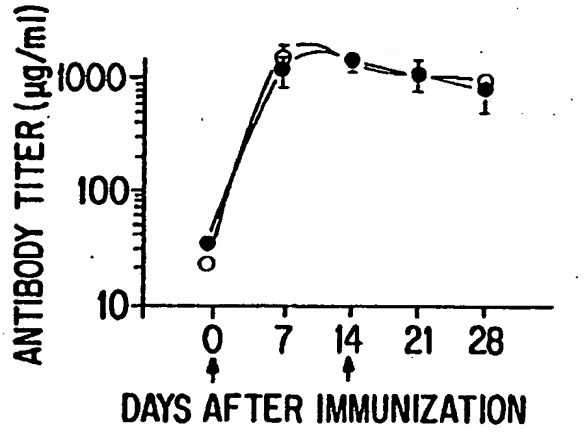

FIG. 6E

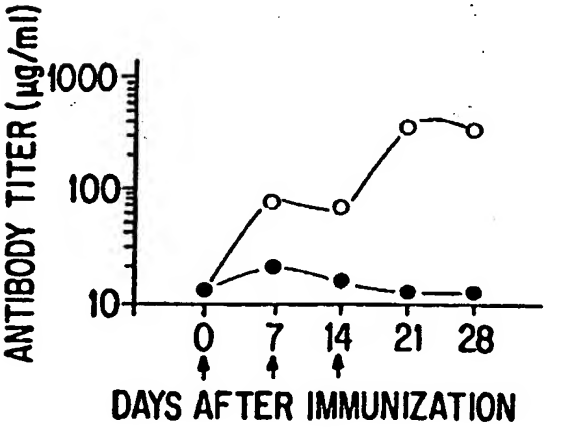

FIG. 6F

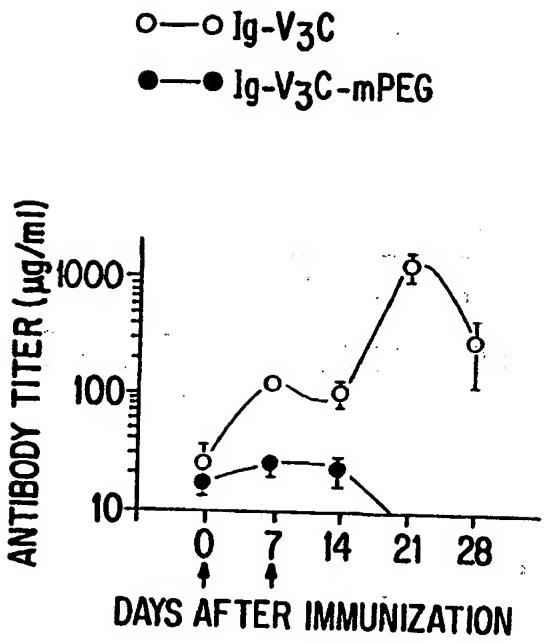
o—o Ig-V3C
●—● Ig-V3C-mPEG
ANTIBODY TITER (µg/ml)
1000
100
10
0
7
14
21
28
DAYS AFTER IMMUNIZATION

FIG. 6G

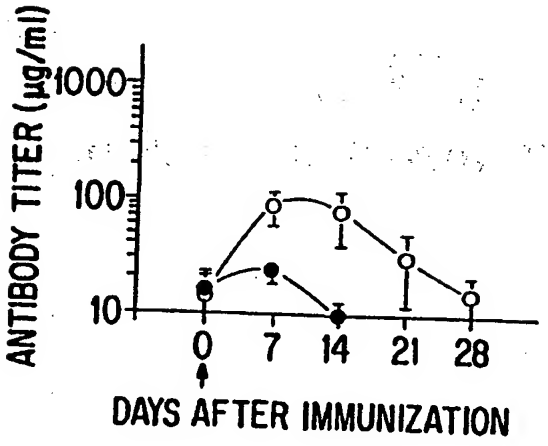
ANTIBODY TITER (µg/ml)
1000
100
10
0
7
14
21
28
DAYS AFTER IMMUNIZATION

FIG. 6H

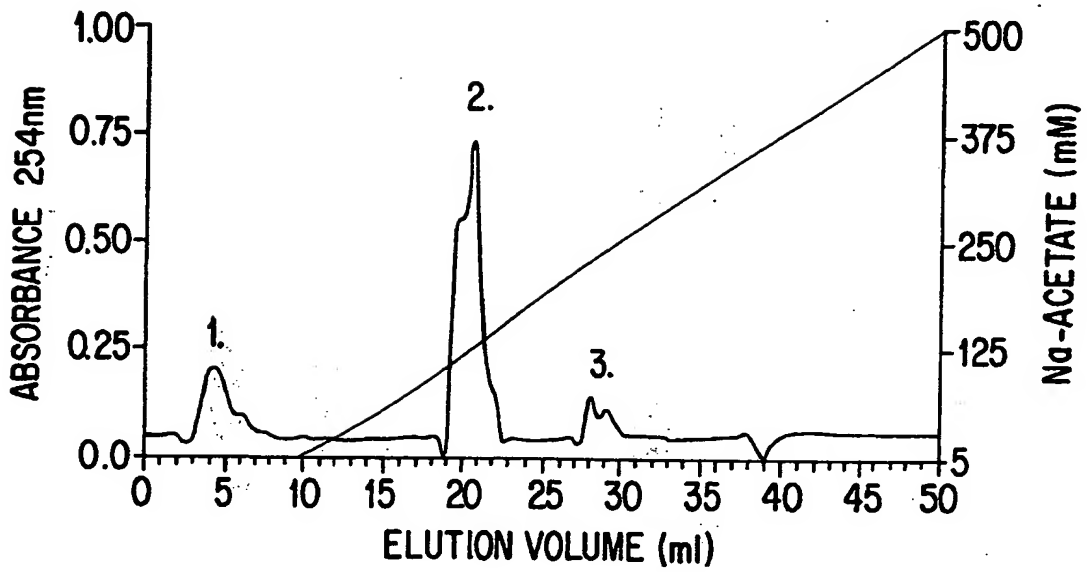
1.00
0.75
0.50
0.25
0.0
ABSORBANCE 254nm
1.
2.
3.
500
375
250
125
5
Na-ACETATE (mM)
0 5 10 15 20 25 30 35 40 45 50
ELUTION VOLUME (ml)

FIG. 7A

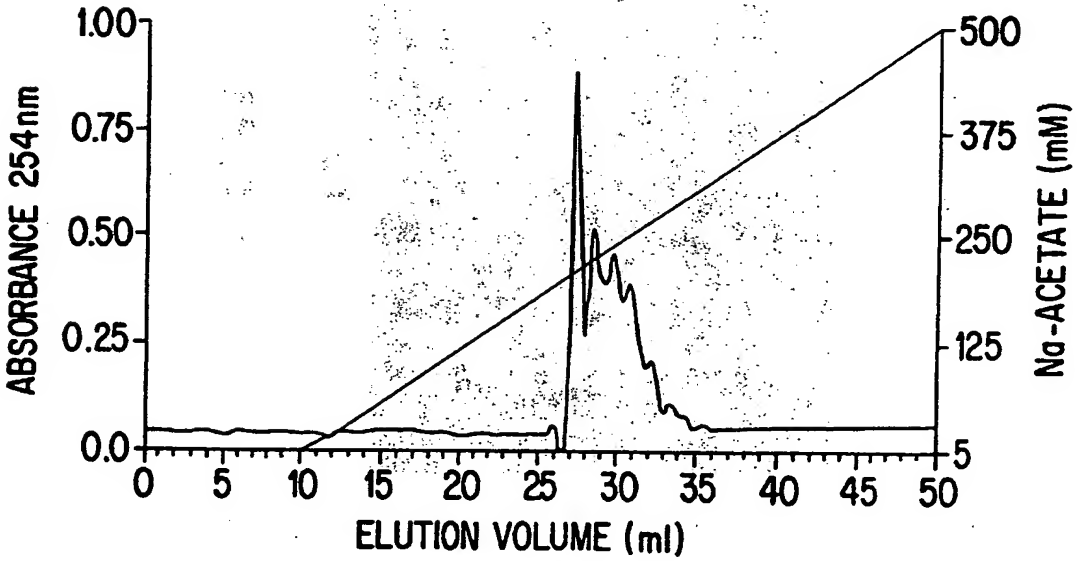
1.00
0.75
0.50
0.25
0.0
ABSORBANCE 254nm
500
375
250
125
5
Na-ACETATE (mM)
0 5 10 15 20 25 30 35 40 45 50
ELUTION VOLUME (ml)

FIG. 7B

BEST AVAILABLE COPY

FIG. 8B

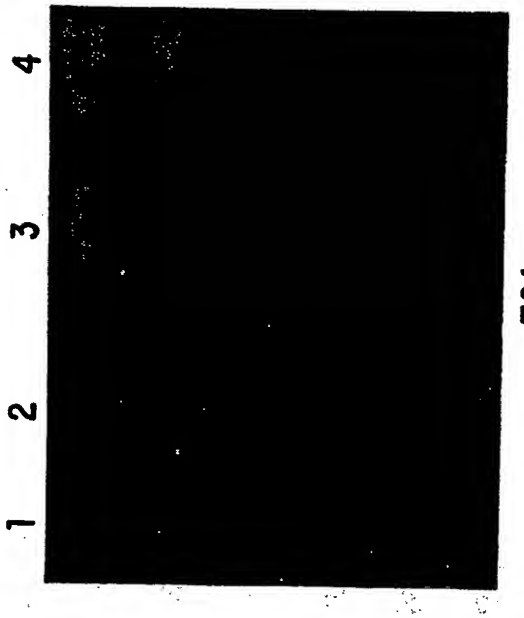

FIG. 8A

BEST AVAILABLE COPY

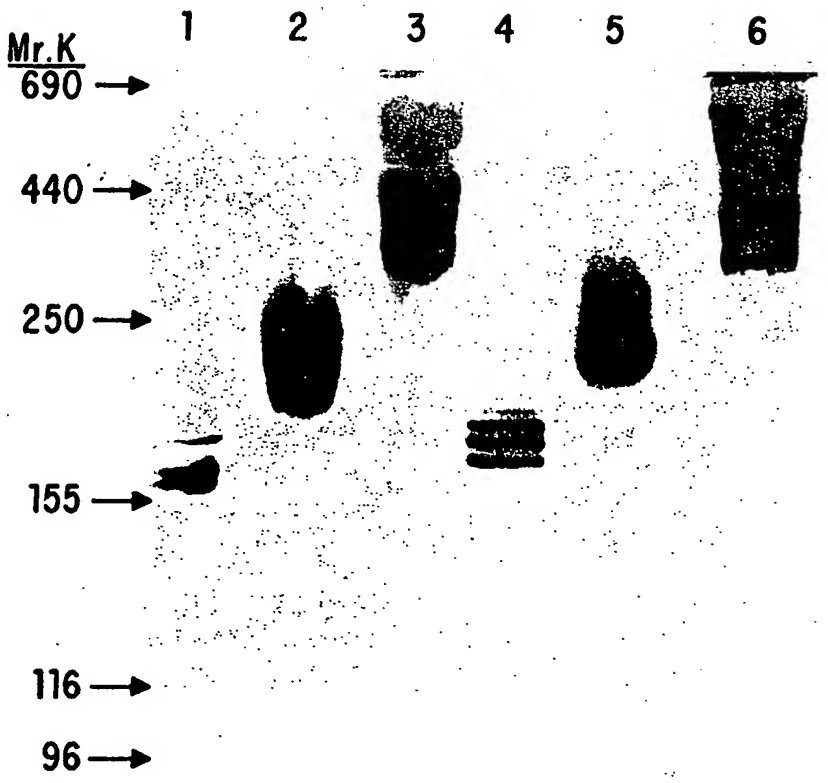

FIG. 9

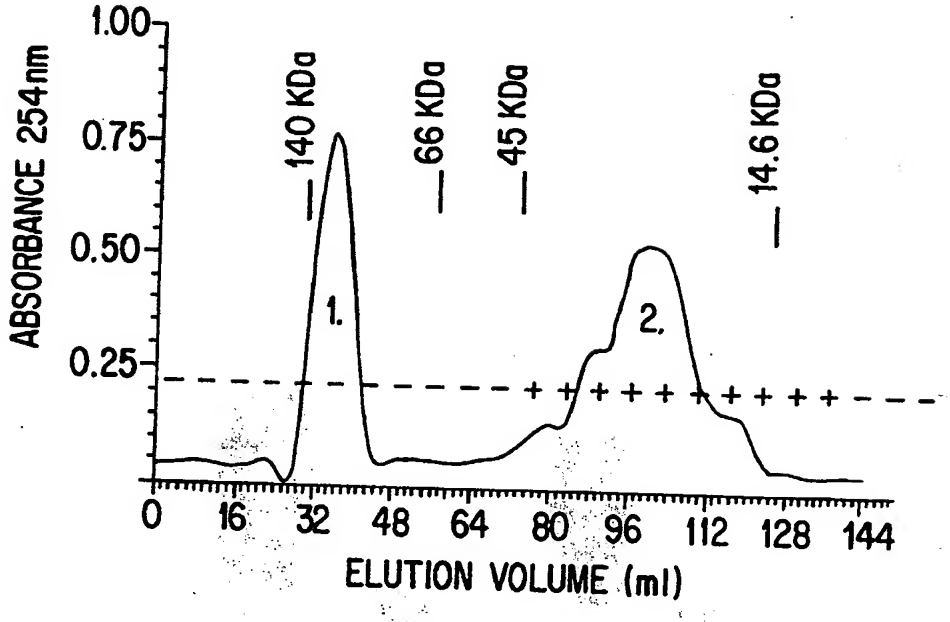

FIG. 10A

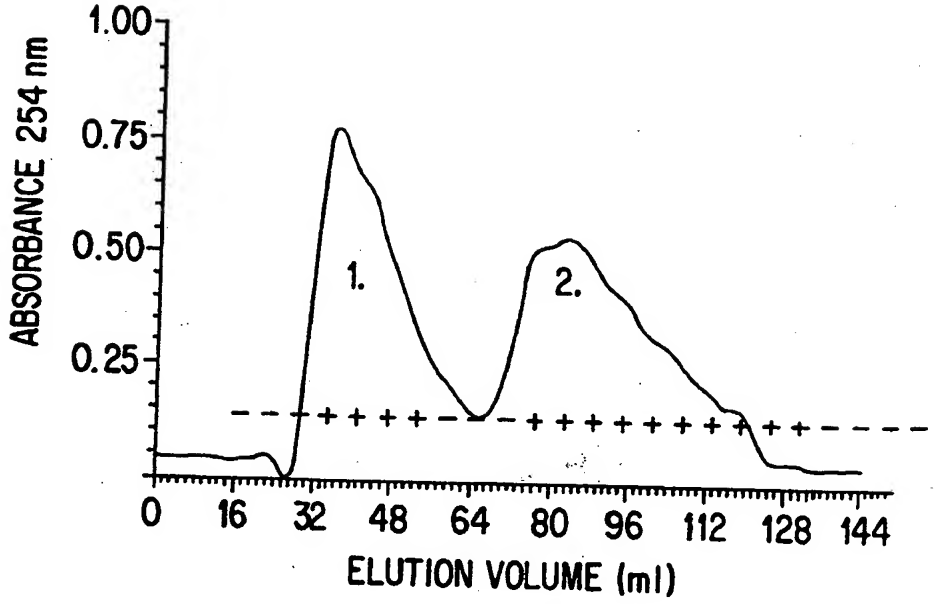

FIG. 10B

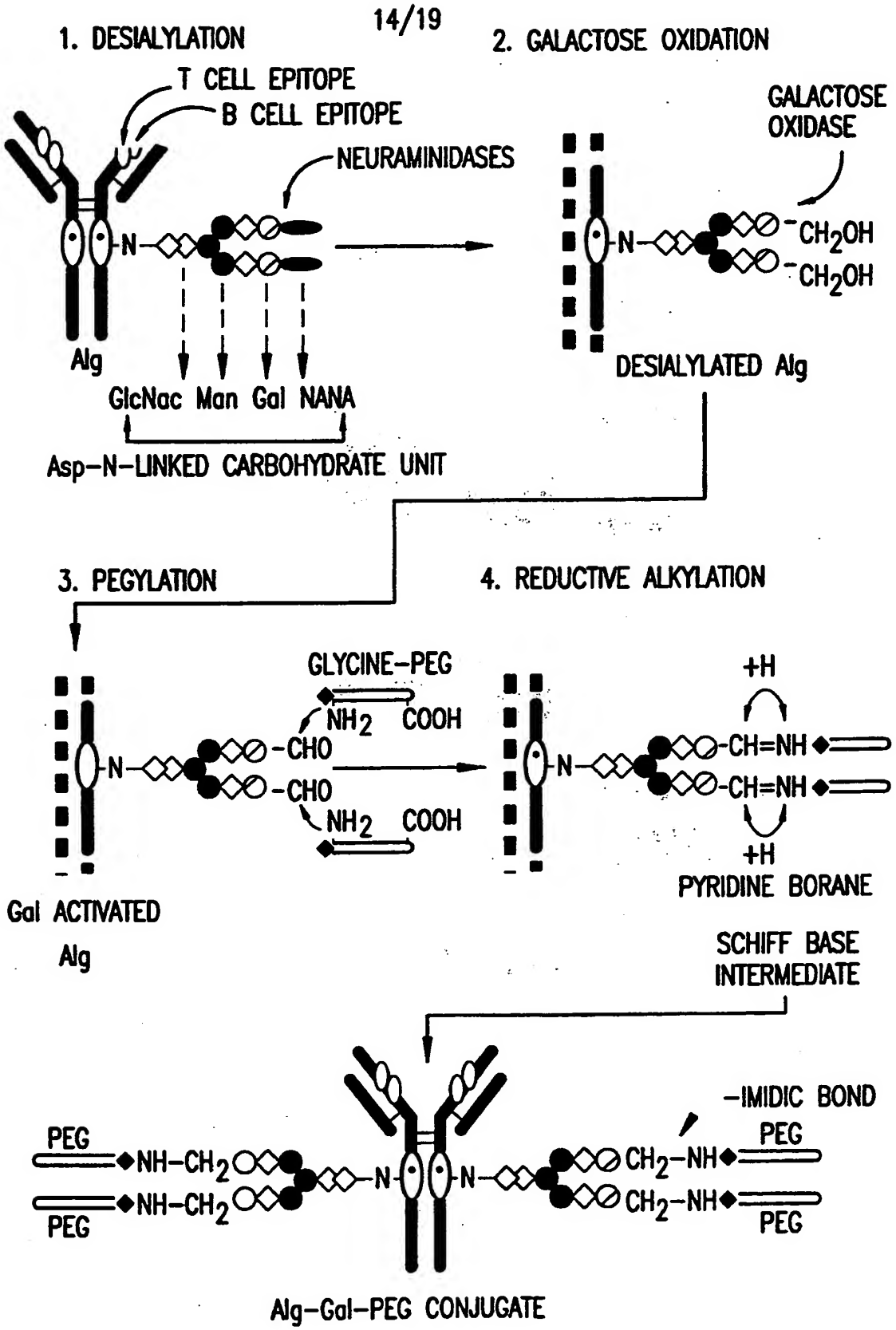
1. DESIALYLATION
T CELL EPITOPE
B CELL EPITOPE
NEURAMINIDASES
Alg
GlcNac Man Gal NANA
Asp-N-LINKED CARBOHYDRATE UNIT
2. GALACTOSE OXIDATION
GALACTOSE OXIDASE
DESIALYLATED Alg
3. PEGYLATION
GLYCINE-PEG
Gal ACTIVATED Alg
4. REDUCTIVE ALKYLATION
+H
PYRIDINE BORANE
SCHIFF BASE INTERMEDIATE
-IMIDIC BOND
PEG
Alg-Gal-PEG CONJUGATE

FIG. 11

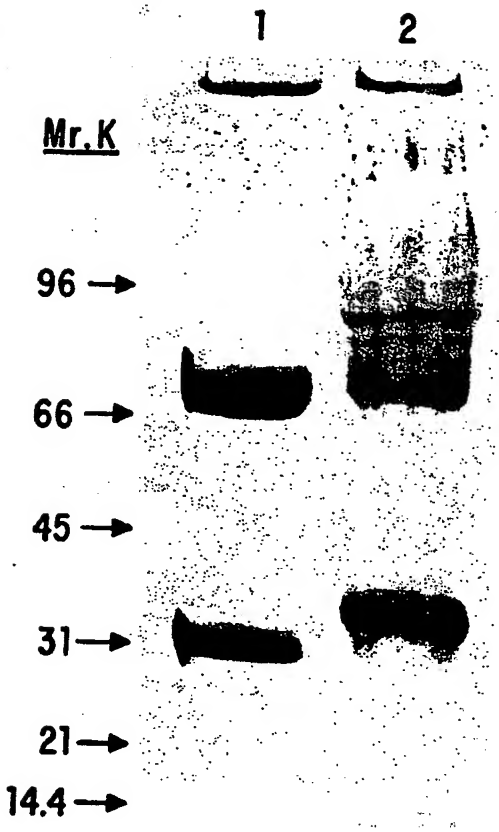

FIG. 12

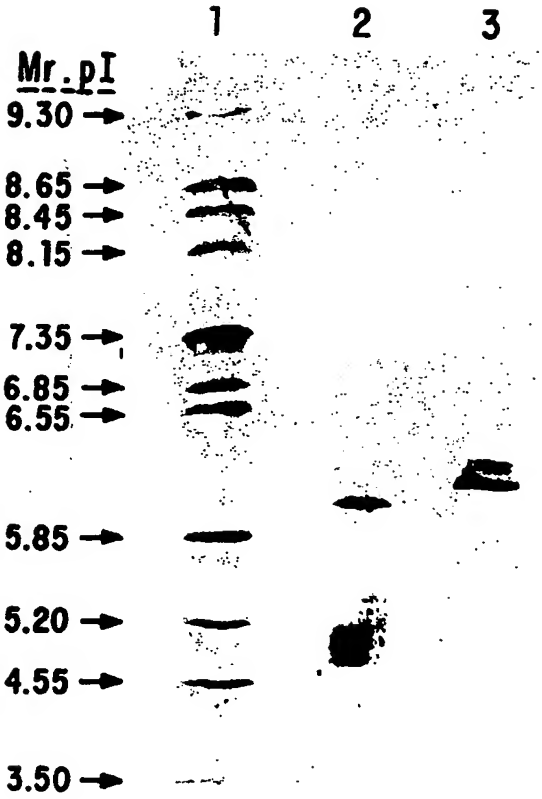

FIG. 13

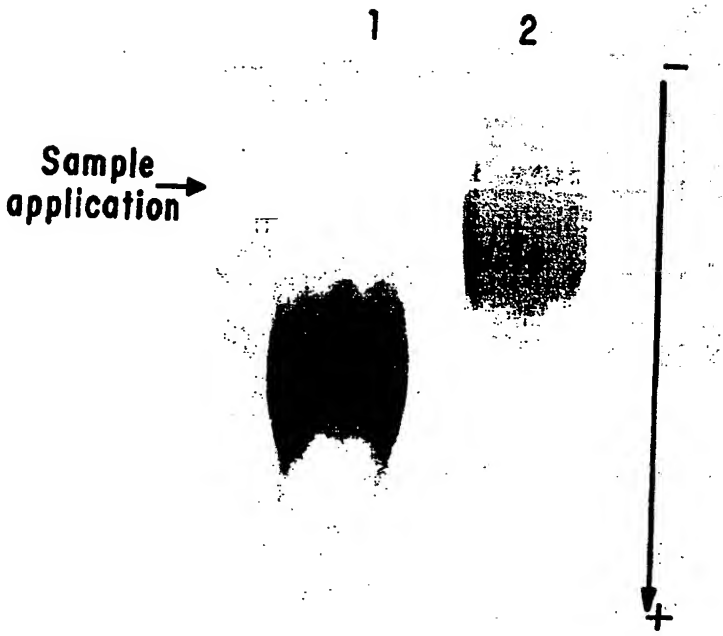

FIG. 14

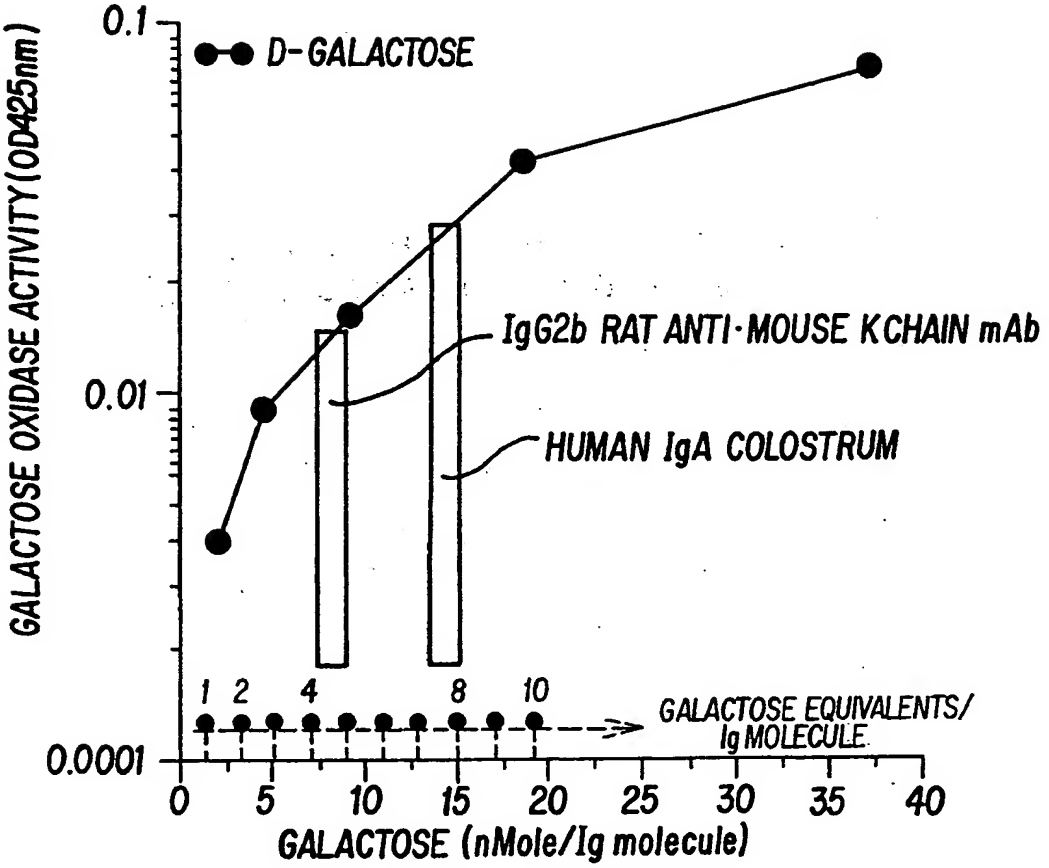
0.1
0.01
0.0001
GALACTOSE OXIDASE ACTIVITY (OD425nm)
D-GALACTOSE
IgG2b RAT ANTI-MOUSE KCHAIN mAb
HUMAN IgA COLOSTRUM
1 2 4 8 10
GALACTOSE EQUIVALENTS/ Ig MOLECULE
0 5 10 15 20 25 30 35 40
GALACTOSE (nMole/Ig molecule)

FIG. 15

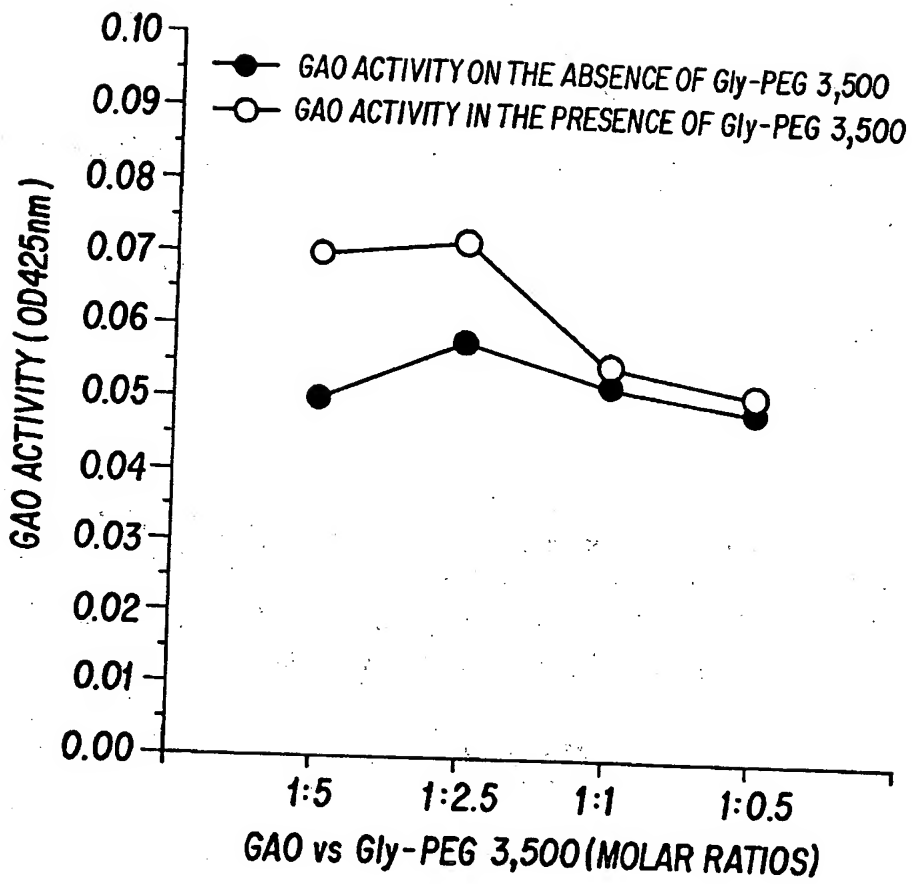
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
GAO ACTIVITY (OD425nm)
GAO ACTIVITY ON THE ABSENCE OF Gly-PEG 3,500
GAO ACTIVITY IN THE PRESENCE OF Gly-PEG 3,500
1:5
1:2.5
1:1
1:0.5
GAO vs Gly-PEG 3,500 (MOLAR RATIOS)

FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08995

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/00, 1/107, 1/13, 16/00; C12P 21/00.
US CL :435/68.1; 530/387.1, 391.1, 402, 403
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/68.1; 530/387.1, 391.1, 402, 403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRUMEANU et al. Derivatization with monomethoxypolyethylene glycol of Igs expressing viral epitopes obviates adjuvant requirements. The Journal of Immunology. 01 April 1995, Vol. 154, pages 3088-3095, see entire document.	1-18
Y	Zalipsky et al. 'Use of Functionalized Poly(Ethylene Glycol)s for Modification of Polypeptides.' In: Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications. Edited by J. Milton Harris. New York: Plenum Press, 1992, pages 347-370. See page 358, section 21.3.5.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 SEPTEMBER 1996	Date of mailing of the international search report 30 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ROBERT D. BUDENS Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

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